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(57) Abstract

This invention relates to chemical synthesis of 2'-*O*-methyl, 3'-*O*-methyl and 5'-*O*-methyl nucleosides, incorporation of novel chemical modifications in enzymatic nucleic acid molecules and improved methods for the synthesis of enzymatic nucleic acid molecules.

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SYNTHESIS OF METHOXY NUCLEOSIDES AND ENZYMATIC NUCLEIC ACID MOLECULES

Background of the Invention

This invention relates to the chemical synthesis of 2'-*O*-methyl, 3'-*O*-methyl and 5'-*O*-methyl nucleosides, incorporation of novel chemical modifications in enzymatic nucleic acid molecules and improved methods for the synthesis of enzymatic nucleic acid molecules.

In one aspect the invention features chemical synthesis of 2'-*O*-methyl, 3'-*O*-methyl and 5'-*O*-methyl nucleosides. The following is a brief description of synthesis of methoxy nucleosides. This summary is not meant to be complete but is provided only for understanding of the invention that follows. This summary is not an admission that all of the work described below is prior art to the claimed invention.

Sugar modifications, such as 2'-*O*-methyl, have been discovered in a variety of naturally occurring RNA (*e.g.*, tRNA, mRNA, rRNA; reviewed by Hall, 1971 *The Modified Nucleosides in Nucleic Acids*, Columbia University Press, New York; Limbach *et al.*, 1994 *Nucleic Acids Res.* 22, 2183). In an attempt to understand the biological significance, structural and thermodynamic properties, and nuclease resistance of these sugar modifications in nucleic acids, several investigators have chemically synthesized nucleosides, nucleotides and phosphoramidites of various sugar modifications and incorporated them into oligonucleotides. There are several reports in the literature describing the synthesis of 2'-*O*-methyl nucleosides, 2'-*O*-methyl nucleotides, 2'-*O*-methyl phosphoramidites and oligonucleotides containing 2'-*O*-methyl substitutions (Broom and Robins, 1965 *J. Am. Chem. Soc.* 87, 1145; Martin *et al.*, 1968 *Biochemistry*, 7, 1406; Robins *et al.*, 1974 *J. Org. Chem.* 39, 1891; Inoue *et al.*, 1987 *Nucleic Acids Res.* 15, 6131; Cotten *et al.*, 1991 *Nucleic Acids Res.* 19, 2629; Andrews *et al.*, 1994 *J. Heterocyclic Chem.*

31, 765; Beigelman *et al.*, 1995 *Nucleosides & Nucleotides* 14, 421; Sproat *et al.*, 1990 *Nucleic Acids Res.* 18, 41).

Broom and Robins, 1965 *J. Am. Chem. Soc.* 87, 1145 and Martin *et al.*, 1968 *Biochemistry*, 7, 1406, describe the synthesis of 2'-*O*-methyl ribonucleotides involving mono-methylation of a 2',3'-cis-diol system of a ribonucleoside with diazomethane. This procedure gives rise to a mixture of 2'- and 3'-*O*-methyl nucleosides in 20-40% combined yield. The two isomers are then separated by ion-exchange chromatography.

Robins *et al.*, 1974 *J. Org. Chem.* 39, 1891, describe the treatment of a methanolic solution of uridine with diazomethane (in glyme) in the presence of stannous chloride dihydrate (in methanol) to synthesize 2'-*O*-methyluridine (58% yield). This reaction also yielded a significant fraction (28%) of 3'-*O*-methyluridine which is purified away from the 2'-*O*-methyl form by chromatography.

Inoue, *Japanese Patent Publication No.* 61291595 and Inoue *et al.*, 1987 *Nucleic Acids Res.* 15, 613, describe a process for the synthesis of 2'-*O*-methyl ribonucleosides involving alkylation of 3',5'-*O*-(tetraisopropyldisiloxane-1, 3-diyl) (TIPDS)-ribonucleosides with methyl iodide. Inoue *et al.*, state that (page 6133, second main paragraph):

> "Treatment of 3',5'-*O*-TIDPS-uridine (1) with benzoyl chloride...in N,N-dimethylacetamide in the presence of triethylamine...selectively gave the N^3-benzoylated derivative (2) in 70.5% yield. Then, 2 was treated with CH_3I...in benzene in the presence of Ag_2O...at 40°C overnight to give the N^3-benzoyl-2'-*O*-methyl derivative (3, 84.5%). Debenzoylation of 3 with dil. NH_4OH followed by removal of TIPDS group with 0.5N HCl afforded 2'-*O*-methyluridine...in 84% yield."

Srivastava and Roy, *US Patent No. 5,214,135*, describe the synthesis of 2-*O*-methyl nucleosides using an approach similar to Inoue *et al.*, *supra*, except that the reaction with methyl iodide/silver oxide was carried out at 25°C for 24-46 hr with an 80-86% yield. This reaction, similar to the one described by Inoue *et al.*, *supra*, also gave rise to the 3'-*O*-methyl isomer in 6-8% yield.

Parmentier *et al.*, 1994 *Tetrahedron* 50, 5361, describe a convergent synthesis of 2'-*O*-methyl uridine. This procedure uses a multi-step process involving– "a facile obtention of the 2'-*O*-methyl sugar synthon using totally selective and efficient methylation conditions;...a stereoselective high-yield condensation with an uracil derivative, yielding the desired β-form with a satisfactory anomeric excess." (page 5361, fifth paragraph).

Chanteloup and Thuong, 1994 *Tetrahedron Letters* 35, 877, describe synthesis of 2'-*O*-alkyl ribonucleosides using trichloroacetimidate D-ribofuranosides as ribosyl donors. They state in the abstract on page 877–

> "Trichloroacetimidate-2-*O*-alkyl-3,5-*O*-TIPS-β-D-ribofuranoside glycosylates silylated nucleobases in a fast high-yielding and stereoselective reaction promoted by trimethylsilyl trifluoromethanesulfonate. This method has been applied to the synthesis of 2'-*O*-alkyl ribonucleosides further transformed to building blocks ready for oligo(2'-*O*-alkyl)ribonucleotide construction."

Beigelman *et al.*, 1995 *Nucleosides & Nucleotides* 14, 421, describe three different approaches to the synthesis of 2'-*O*-methyl nucleosides. They state that–

Method 1:

> " Retrosynthetic analysis showed that 3-*O*-alkylated derivatives of 1,2:5,6-di-*O*-isopropylidene(IP)-α-D-allofuranose (1) could be transformed to the related 2'-*O*-alkyl ribofuranosides by selective degradation of the C1-C2 bond with subsequent cyclization of the generated C2-formyl group to the C5-OH." (Page 421, third paragraph)

Method 2:

> "The 3'-*O*-TBDMS-derivatives of protected ribonucleosides are byproducts obtained during the preparation of 2'-*O*-TBDMS derivatives - key building blocks in oligoribonucleotide synthesis. At the same time, 3'-*O*-TBDMS-isomers could be useful starting compounds in the preparation of 2'-*O*-methyl-3'-*O*-phosphoramidites. We explored this possibility on cytidine derivative **14**. Reaction of 3'-*O*-TBDMS-5'-*O*-DMT-N^4-*i*-Bu-cytidine (**14**) with Ag_2O-CH_3I using a modified method of Ohtsuka *et*

al. (*supra*) yielded 3'-*O*-TBDMS-5'-*O*-DMT-N^4-*i*-Bu-2'-*O*-methyl cytidine (**15**) in 26% yield. The 2'-*O*-TBDMS isomer **16** was also obtained (22% yield) along with the starting 3'-*O*-isomer (18%). When 2'-*O*-TBDMS-5'-*O*-DMT-N^4-*i*-Bu-cytidine (**16**) was subjected to the same reaction conditions, the same mixture of products was obtained. These results show that under the above reaction conditions migration of the TBDMS group accompanies the methylation reaction and methylation takes place selectively at the 2'-OH position." (Page 422, second full paragraph)

Method 3:

"Among different methods of indirect introduction of a methyl group, the use of 1-alkylthioalkyl intermediates seems to be the most promising. Although methods of synthesis of methylthiomethyl ethers of nucleosides and carbohydrates are well developed, their transformation into a methyl group sometimes requires additional steps. We were interested in the testing of more reactive methylthiophenyl ethers as precursors for methyl ethers. We found that methylthiophenyl ethers could be smoothly introduced by treating appropriately protected nucleosides or carbohydrates with PhSMe/Bz_2O_2 in the presence of DMAP. Nucleoside **19** afforded methythiophenyl ether **20** in 65-70% yield, and α-ribofuranose **21** was transformed into β-furanose **22** in 60% yield. Different attempts to radically (Bu_3SnH, Bz_2O_2) reduce the thiophenyl group of furanose **22** were not successful, providing only starting material. However, under the same conditions, nucleoside **20** afforded 2'-*O*-Me derivative **24** in 70% yield.

Haga *et al.*, 1972 *Carbohydrate Res.* 21, 440 describe a "facile route" to the synthesis of 2- and 3-*O*-methyl-D-ribose from 3-*O*-methyl-D-allose.

Nair *et al.*, 1982, *Synthesis* 8, 670, describes the modification of Nucleic acid bases via radical intermediates.

Leonard *et al.*, 1992, *Nucleosides & Nucleotides*, 11, 1201, describe a method for the preparation of protected 2'-*O*-methylguanosine. This procedure is distinct from the one described in the instant invention.

Wagner *et al.*, 1991, *Nucleic Acids Res.*, 19, 5965, describes a method for alkylation of ribonucleosides.

In a second aspect, the invention features improved methods for the synthesis of enzymatic nucleic acid molcules.

Ribozymes are nucleic acid molecules having an enzymatic activity which is able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence specific manner. Such enzymatic RNA molecules can be targeted to virtually any RNA transcript, and efficient cleavage achieved in vitro. Kim et al., 84 Proc. Natl. Acad. Sci. USA 8788, 1987; Haseloff and Gerlach, 334 Nature 585, 1988; Cech, 260 JAMA 3030, 1988; and Jefferies et al., 17 Nucleic Acids Research 1371, 1989.

Ribozymes act by first binding to a target RNA. Such binding occurs through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA which acts to cleave the target RNA. Thus, the ribozyme first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After a ribozyme has bound and cleaved its RNA target it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with other RNA sequences by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

Six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. Table I summarizes some of the characteristics of these ribozymes. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA.

Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The general structure of various ribozyme motifs is described by Draper et al. WO 93/23569, at pages 3-4 and in Usman et al., 95/06731 at pages 1-7 hereby incorporated by reference herein in its entirety (including drawings). Other motifs are also known in the art.

The enzymatic nature of a ribozyme is advantageous over other technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the effective concentration of ribozyme necessary to effect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

Jennings et al., US Patent No. 5, 298, 612 describe the use of non-nucleotides to assemble a hammerhead ribozyme lacking a stem II portion.

Draper et al., WO 93/23569 (PCT/US93/04020) describes synthesis of ribozymes in two parts in order to aid in the synthetic process (see, e.g., p. 40).

Usman et al., WO 95/06731, describe enzymatic nucleic acid molecules having non-nucleotides within their structure. Such non-nucleotides can be used in place of nucleotides to allow formation of an enzymatic nucleic acid.

In a third aspect, the invention features enzymatic nucleic acid molecules with chemical modifications at the 5'- and/or 3'-ends.

Chemically-modified ribozymes can be synthesized which are stable in human serum for up to 260 hours (Beigelman *et al.*, 1995 *supra*) and maintain near *wild type* (chemically unmodified equivalent of modified ribozyme) activity *in vitro*. A number of laboratories have reported that the enhanced cellular efficacy of phosphorothioate-substituted antisense molecules. The enhanced efficacy appears to result from either i) increased resistance to 5'-exonuclease digestion (De Clercq *et al.*., 1970 *Virology* **42**, 421-428; Shaw *et al.*., 1991 *Nucleic Acids Res.* **19**, 747-750), ii) intracellular localization to the nucleus (Marti *et al.*, 1992 *Antisense Res. Dev.* **2**, 27-39), or iii) sequence-dependent non-specific effects (Gao *et al.*, 1992 *Molec.. Pharmac.* **41**, 223-229; Bock *et al.*, 1992 *Nature* **355**, 564-566; and Azad, *et al.*, 1993 *Antimicrob. Agents Chemother.* **37**, 1945-1954) which are not manifested in nonthioated molecules. Many effects of thioated compounds are probably due to their inherent tendency to associate non-specifically with cellular proteins such as the Sp1 transcription factor (Perez *et al.*, 1994 *Proc. Natl Acad Sci. U.S. A.* **91**, 5957-5961). Chemical modification of enzymatic nucleic acids that provide resistance to cellular 5'-exonuclease and 3'-exonuclease digestion without reducing the catalytic activity or cellular efficacy will be important for *in vitro* and *in vivo* applications of ribozymes.

Modification of oligonucleotides with a 5'-amino group offered resistance against 5'-exonuclease digestion *in vitro* (Letsinger & Mungall, 1970 *J. Org. Chem.* **35**, 3800-3803).

Heidenreich *et al.*, 1993 *FASEB J.* 7, 90 and Lyngstadaas *et al.*, 1995 *EMBO. J.* 14, 5224, mention that hammerhead ribozymes with terminal phosphorothioate linkages can increase resistance against cellular exonucleases.

Seliger *et al.*, *Canadian Patent Application* No. CA 2,106,819 and *Prog. Biotechnol.* 1994, 9 (EC B6: Proceedings Of The 6th European Congress On Biotechnology, 1993, Pt. 2), 681-4 describe "oligoribonucleotide and ribozyme analogs with terminal 3'-3' and/or 5'-5' internucleotide linkages".

The references cited above are distinct from the presently claimed invention since they do not disclose and/or contemplate the the methods used for the synthesis of methoxy nucleosides and enzymatic nucleic acid molecules of the instant invention. Further none of the references cited above disclose the specific chemical modifications used to stabilize the termini of enzymatic nucleic acid molecules.

Summary of the Invention

Chemical synthesis of 2'-*O*-methyl, 3'-*O*-methyl and 5'-*O*-methyl nucleosides

It has been postulated (Ueda, in *Chemistry of Nucleosides and Nucleotides* ed. L.B Townsend, v.1 Plenum Press 1988 pp.1-95) that protonation of the N_3 atom of 2,2'-, 2,3' or 2,5'-anhydro pyrimidine nucleosides facilitates anhydro ring opening by different nucleophiles producing, in most cases, nucleoside analogs containing modifications in the carbohydrate portion of the nucleoside. Complexation of the N_3 atom of the above-mentioned anhydro derivatives with Lewis acids [*e.g.* $B(OMe)_3$] would provide the same effect directly or in the case of methanolysis, complexation of the MeOH with Lewis acids would acidify the related proton leading to potential protonation of the N_3 atom of the above-mentioned anhydro derivatives. Applicant investigated methanolysis of 2,2'-, 2,3' or 2,5'-anhydro pyrimidine nucleosides in the presence of a Lewis acid, such as $B(OMe)_3$ and/or BF_3.MeOH. The reaction involving a 2,2'-anhydro-1(β-D-arabinofuranosyl) nucleoside, such as 2,2'-anhydro-1(β-D-arabinofuranosyl) uracil or 2,2'-anhydro-1(β-D-arabinofuranosyl) cytosine, with $B(OMe)_3$ and/or BF_3.MeOH, results in the production of 2'-*O*-methyl nucleosides with a yield of about 90-100%.

By "Lewis Acid" is meant a substance that can accept an electron pair from a base. Examples of Lewis acids are, $B(OCH_3)_3$, BF_3, $AlCl_3$, and SO_3.

In one aspect, the invention features a process for the synthesis of a 2'-*O*-methyl adenosine nucleoside, comprising the step of contacting a solution of N^4-acetyl-5',3'-di-*O*-acetyl-2'-*O*-methyl cytidine with a Lewis acid under conditions suitable for the formation of said nucleoside.

In another aspect, the invention features a process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of: a) methylating 2-amino-6-chloropurine riboside by contacting said 2-amino-6-chloropurine riboside with sodium hydride, dimethylformamide and methyl iodide under conditions suitable for the formation of 2'-*O*-methyl-2-amino-6-chloropurine riboside; b) contacting said 2'-*O*-methyl-2-amino-6-chloropurine riboside with 1,4-diazabicyclo(2.2.2) octane and water under conditions suitable for the formation of said 2'-*O*-methyl guanosine nucleoside in a crude form; and c) purifying said 2'-*O*-methyl guanosine nucleoside from said crude form.

In another aspect, the invention features a process for the synthesis of 2'-*O*-methyl adenosine nucleoside, comprising the steps of: a) methylating 2-amino-6-chloropurine riboside by contacting said 2-amino-6-chloropurine riboside with sodium hydride, dimethylformamide and methyl iodide under conditions suitable for the formation of 2'-*O*-methyl-2-amino-6-chloropurine riboside; b) contacting said 2'-*O*-methyl-2-amino-6-chloropurine riboside with acetic anhydride, 4-dimethylaminopyridine and triethylamine under conditions suitable for the formation of 3',5'-di-*O*-acetyl-2'-*O*-methyl-6-chloro-2-aminopurine riboside; c) deaminating said 3',5'-di-*O*-acetyl-2'-*O*-methyl-6-chloro-2-aminopurine riboside with isoamyl nitrite and tetrahydrofuran to form 3',5'-di-*O*-acetyl-2'-*O*-methyl-6-chloropurine; d) aminating said 3',5'-di-*O*-acetyl-2'-*O*-methyl-6-chloropurine with ammonia to form 2'-*O*-methyl adenosine nucleoside in a crude form; and e) purifying said 2'-*O*-methyl adenosine nucleoside from said crude form.

In yet another aspect, the invention features a process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of: a) contacting 2,6-diaminopurine nucleoside with anhydrous pyridine and tetraisopropyl D-silyl chloride under conditions suitable for the formation of 2,6-diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine; b) methylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine with anhydrous DMF and methyl iodide under conditions suitable for the formation of 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-2'-*O*-methyl-β-D-ribofuranosyl) purine; c) acylating said 2,6-Diamino-9-(3,5-O-

tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine with anhydrous pyridine and isobutyryl chloride under conditions suitable for the formation of *2,6-Diamino-N^2-isobutyryl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-O-methyl-β-D-ribofuranosyl) purine*; d) deaminating and desilylating said *2,6-Diamino-N^2-isobutyryl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine* under conditions suitable for the formation of N^2-isobutyryl-2'-*O*-methyl guanosine nucleoside in a crude form; e) purifying said N^2-isobutyryl-2'-*O*-methyl guanosine nucleoside from said crude form; and f) deblocking said N^2-isobutyryl-2'-*O*-methyl guanosine nucleoside under suitable conditions to form said 2'-*O*-methyl guanosine nucleoside.

In one aspect, the invention features a process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of: a) contacting 2,6-diaminopurine nucleoside with anhydrous pyridine and tetraisopropyl D-silyl chloride (TIPSCl) under conditions suitable for the formation of 2,6-Diamino-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine; b) methylating said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine with anhydrous DMF and methyl iodide under conditions suitable for the formation of 2,6-Diamino-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-*O*-methyl-β-D-ribofuranosyl) purine; c) acylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine with anhydrous pyridine and isopropylphenoxyacetyl chloride under conditions suitable for the formation of *2,6-Diamino-N^2-isopropylphenoxyacetyl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine*; d) deaminating and desilylating said *2,6-Diamino-N^2-isopropylphenoxyacetyl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine* under conditions suitable for the formation of N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside in a crude form; e) purifying said N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside from said crude

form; and f) deblocking said N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside under suitable conditions to form said 2'-*O*-methyl guanosine nucleoside.

In one aspect, the invention features a process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of: a) contacting guanosine with N,N-dimethylformamide dibenzyl acetal under conditions suitable for the formation of N1-benzyl guanosine; b) methylating said N1-benzyl guanosine by contacting said N1-benzyl guanosine with silver acetylacetonate, trimethylsulphonium hydroxide and dimethylformamide under conditions suitable for the formation of N1-benzyl-2'-*O*-methyl guanosine in a crude form; c) purifying said N1-benzyl-2'-*O*-methyl guanosine from said crude form; d) removing the N1-benzyl protection from said N1-benzyl-2'-*O*-methyl guanosine by contacting said N1-benzyl-2'-*O*-methyl guanosine with sodium naphthalene under conditions suitable for the formation of 2'-*O*-methyl guanosine nucleoside in a crude form; and e) purifying said 2'-*O*-methyl guanosine from said crude form.

In yet another aspect, the invention features a process for the synthesis of 2'-*O*-methyl adenosine nucleoside, comprising the steps of: a) methylating adenosine by contacting said adenosine with dimethylformamide, silver acetylacetonate and trimethylsulphonium hydroxide under conditions suitable for the formation of 2'-*O*-methyl adenosine in a crude form; and b) purifying said 2'-*O*-methyl adenosine from said crude form.

In one aspect, the invention also features a process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of: a) contacting guanosine with N,N-dimethylformamide dibenzyl acetal under conditions suitable for the formation of N1-benzyl guanosine; b) methylating said N1-benzyl guanosine by contacting said N1-benzyl guanosine with magnesium acetylacetonate, trimethylsulphonium hydroxide and dimethylformamide under conditions suitable for the formation of N1-benzyl-2'-*O*-methyl guanosine in a crude form; c) purifying said N1-benzyl-2'-*O*-methyl guanosine from said crude form; d) removing the N1-benzyl protection from said N1-benzyl-2'-*O*-methyl guanosine by contacting said N1-benzyl-2'-*O*-methyl guanosine with sodium naphthalene under conditions suitable for the

formation of 2'-*O*-methyl guanosine nucleoside in a crude form; and e) purifying said 2'-*O*-methyl guanosine nucleoside from said crude form.

In one aspect, the invention features a process for the synthesis of 2'-*O*-methyl adenosine nucleoside, comprising the steps of: a) methylating adenosine by contacting said adenosine with dimethylformamide, magnesium acetylacetonate and trimethylsulphonium hydroxide under conditions suitable for the formation of 2'-*O*-methyl adenosine in a crude form; and b) purifying said 2'-*O*-methyl adenosine from said crude form.

In one aspect, the invention features a process for the synthesis of 2'-*O*-methyl adenosine nucleoside, comprising the steps of: a) methylating adenosine by contacting said adenosine with dimethylformamide, strontium acetylacetonate and trimethylsulphonium hydroxide under conditions suitable for the formation of 2'-*O*-methyl adenosine in a crude form; and b) purifying said 2'-*O*-methyl adenosine from said crude form.

In one aspect, the invention features a process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of: a) contacting 2,6-diaminopurine nucleoside with anhydrous pyridine and TIPSCl under conditions suitable for the formation of 2,6-diamino-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine; b) methylating said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine with anhydrous DMF and methyl iodide under conditions suitable for the formation of 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine; c) acylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine with anhydrous pyridine and isobutyryl chloride under conditions suitable for the formation of *2,6-Diamino-N^2-isobutyryl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine*; d) deaminating and desilylating said *2,6-Diamino-N^2-isobutyryl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-O-methyl-β-D-ribofuranosyl) purine* under conditions suitable for the formation of N2-

isobutyryl-2'-*O*-methyl guanosine nucleoside in a crude form; and e) purifying said N2-isobutyryl-2'-*O*-methyl guanosine nucleoside from said crude form.

In yet another embodiment, the invention features a process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of: a) contacting 2,6-diaminopurine nucleoside with anhydrous pyridine and TIPSCl under conditions suitable for the formation of 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine; b) methylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine with anhydrous DMF and methyl iodide under conditions suitable for the formation of 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine; c) acylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-*O*-methyl-β-D-ribofuranosyl) purine with anhydrous pyridine and isopropylphenoxyacetyl chloride under conditions suitable for the formation of *2,6-Diamino-N^2-isopropylphenoxyacetyl-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-O-methyl-β-D-ribofuranosyl) purine*; d) deaminating and desilylating said *2,6-Diamino-N^2-isopropylphenoxyacetyl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine* under conditions suitable for the formation of N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside in a crude form; and e) purifying said N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside from said crude form.

This invention features an improved and economical synthetic method for the preparation of 2'-*O*-methyl nucleosides in high yield. The method is not only cost efficient, but can be scaled up to several hundred gram quantities. The method generally utilizes inexpensive commercially available 2,2'-anhydro-1(β-D-arabinofuranosyl)base, such as 2,2'-anhydro-1(β-D-arabinofuranosyl)uracil or 2,2'-anhydro-1(β-D-arabinofuranosyl)cytosine, as a starting material which is converted in a one or two step reaction sequence to 2'-*O*-methyl nucleosides with a yield of about 90-100%.

The 2'-*O*-methyl or 3'-*O*-methyl nucleosides can be used for chemical synthesis of nucleotides, nucleotide-tri-phosphates and/or phosphoramidites as a building block for selective incorporation into oligonucleotides. These oligonucleotides can be used as an antisense molecule, 2-5A antisense chimera, triplex molecule or as an enzymatic nucleic acid molecule. The oligonucleotides can also be used as probes or primers for synthesis and/or sequencing of RNA or DNA.

By "antisense" it is meant a non-enzymatic nucleic acid molecule that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm *et al.*, 1993 *Nature* 365, 566) interactions and alters the activity of the target RNA (for a review see Stein and Cheng, 1993 *Science* 261, 1004).

By "2-5A antisense chimera" it is meant, an antisense oligonucleotide containing a 5' phosphorylated 2'-5'-linked adenylate residues. These chimeras bind to target RNA in a sequence-specific manner and activate a cellular 2-5A-dependent ribonuclease which, in turn, cleaves the target RNA (Torrence *et al.*, 1993 *Proc. Natl. Acad. Sci. USA* 90, 1300).

By "triplex DNA" it is meant an oligonucleotide that can bind to a double-stranded DNA in a sequence-specific manner to form a triple-strand helix. Formation of such triple helix structure has been shown to inhibit transcription of the targeted gene (Duval-Valentin *et al.*, 1992 *Proc. Natl. Acad. Sci. USA* 89, 504).

By "enzymatic nucleic acid" it is meant a nucleic acid molecule capable of catalyzing reactions including, but not limited to, site-specific cleavage and/or ligation of other nucleic acid molecules, cleavage of peptide and amide bonds, and trans-splicing.

In preferred embodiments, the invention features a method for chemical synthesis of 2'-*O*-methyl or 3'-*O*-methyl nucleosides in which 2,2'-anhydro-1(β-D-arabinofuranosyl) cytosine, 2,2'-anhydro-1(β-D-arabinofuranosyl) uracil, 2,3'-anhydro-1(β-D-arabinofuranosyl) uracil, or 2,3'-anhydro-1(β-D-arabinofuranosyl) cytidine is used as the starting material, and wherein said starting material is reacted with a Lewis acid.

Another preferred embodiment of the invention features a method for chemical synthesis of 5'-*O*-methyl pyrimidine nucleoside in which 2,5'-anhydro-1(β-D-arabinofuranosyl) pyrimidine is used as the starting material and is reacted with a Lewis acid.

In yet another preferred embodiment, the invention features novel processes for the synthesis of 2'-*O*-methyl purine nucleosides.

Another preferred embodiment of the invention features a method for the chemical synthesis of Purines.

Chemical linkage of ribozyme portions:

This invention relates to improved methods for synthesis of enzymatic nucleic acids and, in particular, hammerhead and hairpin motif ribozymes. This invention is advantageous over iterative chemical synthesis of ribozymes since the yield of the final ribozyme can be significantly increased. Rather than synthesizing, for example, a 37mer hammerhead ribozyme, two partial ribozyme portions, e.g., a 20mer and a 17mer, can be synthesized in significantly higher yield, and the two reacted together to form the desired enzymatic nucleic acid.

Referring to Fig. 16, the strategy involved is shown for a hammerhead ribozyme where each n or n' is independently any desired nucleotide or non-nucleotide, each filled-in circle represents pairing between bases or other entities, and the solid line represents a covalent bond. Within the structure each n and n' may be a ribonucleotide, a 2'-methoxy-substituted nucleotide, or any other type of nucleotide which does not significantly affect the desired enzymatic activity of the final product (see Usman et al., supra). In the particular embodiment shown, which is not limiting in this invention, five ribonucleotides are provided at rG5, rA6, rG8, rG12, and rA15.1. U4 and U7 may be abasic (i.e., lacking the uridine moiety) or may be ribonucleotides, 2'-methoxy substituted nucleotides, or other such nucleotides. a9, a13, and a14 are preferably 2'-methoxy or may have other substituents. The synthesis of this hammerhead ribozyme is performed by synthesizing a 3' and a 5' portion as shown in a lower part of Fig. 16. Each 5' and 3' portion has a chemically reactive group X and Y, respectively. Non-limiting examples of such

chemically reactive groups are provided in Fig. 17. These groups undergo chemical reactions to provide the bonds shown in Fig. 17. Thus, the X and Y can be used, in various combinations, in this invention to form a chemical linkage between two ribozyme portions.

Thus, in a one aspect the invention features a method for synthesis of an enzymatically active nucleic acid (as defined by Draper, supra) by providing a 3' and a 5' portion of that nucleic acid, each having independently chemically reactive groups at the 5' and 3' positions, respectively. The reaction is performed under conditions in which a covalent bond is formed between the 3' and 5' portions by those chemically reactive groups. The bond formed can be, but is not limited to, either a disulfide, morpholino, amide, ether, thioether, amine, a double bond, a sulfonamide, carbonate, hydrazone or ester bond. The bond is not the natural bond formed between a 5' phosphate group and a 3' hydroxyl group which is made during normal synthesis of an oligonucleotide. In other embodiments, more than two portions can be linked together using pairs of X and Y groups which allow proper formation of the ribozyme.

By "chemically reactive group" is simply meant a group which can react with another group to form the desired bonds. These bonds may be formed under any conditions which will not significantly affect the structure of the resulting enzymatic nucleic acid. Those in the art will recognize that suitable protecting groups can be provided on the ribozyme portions.

In preferred embodiments the nucleic acid has a hammerhead motif and the 3' and 5' portions each have chemically reactive groups in or immediately adjacent to the stem II region (see Fig. 1). The stem II region is evident in Fig. 1 between the bases termed a9 and rG12. The C and G within this stem defines the end of the stem II region. Thus, any of the n or n' moieties within the stem II region can be provided with a chemically reactive group. As is evident from this structure, the chemically reactive groups need not be provided in the solid line portion but can be provided at any of the n or n'. In this way the length of each of the 5' and 3' portions can vary by several bases.

In other preferred embodiments, the chemically reactive group can be, but is not limited to, $(CH_2)_nSH$; $(CH_2)_n$ NHR; $(CH_2)_nX$; ribose; COOH; $(CH_2)_n$ PPh_3; $(CH_2)_n$ SO_2Cl; $(CH_2)_nCOR$; $(CH_2)_nRNH$ or $(CH_2)_n$ OH, where, CH_2 can be replaced by another group which forms linking chain containing various atoms including, but not limited to CH_2, such as methylenes, ether, ethylene glycol, thioethers, double bonds, aromatic groups and others; each n independently is an integer from 0 to 10 inclusive and may be the same or different; each R independently is a proton or an alkyl, alkenyl and other functional groups or conjugates such as peptides, steroids, hoemones, lipids, nucleic acid sequences and others that provides nuclease resistance, improved cell association, improved cellular uptake or interacellular localization. X is halogen, and Ph represents a phenyl ring.

Specifically, an "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino, or SH. The term also includes alkenyl groups which are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, halogen, $N(CH_3)_2$, amino, or SH. The term "alkyl" also includes alkynyl groups which have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino or SH.

Such alkyl groups may also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an

aromatic group which has at least one ring having a conjugated π electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above. Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

In yet other preferred embodiments, the conditions include provision of $NaIO_4$ in contact with the ribose, and subsequent provision of a reducing group such as $NaBH_4$ or $NaCNBH_3$; or the conditions include provision of a coupling reagent.

In a second related aspect, the invention features a mixture of the 5' and 3' portions of the enzymatically active nucleic acids having the 3' and 5' chemically reactive groups noted above.

Those in the art will recognize that while examples are provided of half ribozymes it is possible to provide ribozymes in 3 or more portions. For example, the hairpin ribozyme may be synthesized by inclusion of chemically reactive groups in helix IV and in other helices which are not critical to the enzymatic activity of the nucleic acid.

Enzymatic nucleic acid molecules containing 5'- and/or 3'-cap structures

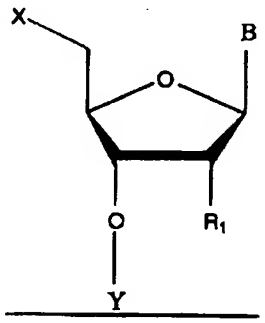
This invention relates to the incorporation of chemical modifications at the 5' and/or 3' ends of nucleic acids, which are particularly useful for enzymatic cleavage of RNA or single-stranded DNA. These terminal

modifications are termed as either a 5'-cap or a 3'-cap depending on the terminus that is modified. Certain of these modifications protect the enzymatic nucleic acids from exonuclease degradation. Resistance to exonuclease degradation can increase the half-life of these nucleic acids inside a cell and improve the overall effectiveness of the enzymatic nucleic acids. These terminal modifications can also be used to facilitate efficient uptake of enzymatic nucleic acids by cells, transport and localization of enzymatic nucleic acids within a cell, and help achieve an overall improvement in the efficacy of ribozymes *in vitro* and *in vivo*.

The term "chemical modification" as used herein refers to any base, sugar and/or phosphate modification that will protect the enzymatic nucleic acids from degradation by nucleases. Non-limiting examples of some of the chemical modifications and methods for their synthesis and incorporation in nucleic acids are described in Figures 28, 29, 32-37 and *infra*.

In a preferred embodiment, chemical modifications of enzymatic nucleic acids are featured that provide resistance to cellular 5'-exonuclease and/or 3'-exonuclease digestion without reducing the catalytic activity or cellular efficacy of these nucleic acids .

In a second aspect, the invention features enzymatic nucleic acids with 5'-end modifications (5'-cap) having the formula:

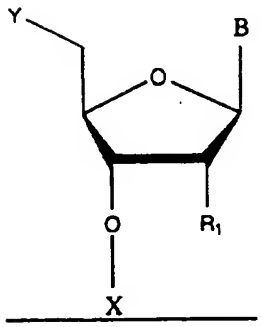


wherein, X represents H, alkyl, amino alkyl, hydroxy alkyl, halo, trihalomethyl [CX_3 (X = Br, Cl, F), N_3, NH_2, NHR, NR_2 [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], NO_2, $CONH_2$, COOR [R=alkyl (C1-

22), acyl (C1-22), substituted (alkyl, amino, alkoxy, halogen, or the like) or unsubstituted aryl], SH, SR [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], OR [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], ONHR [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], 1,4-butanediol phosphate, or ONR_2 [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], PO_4^{2-}, PO_3S^{2-}, $PO_2S_2^{2-}$, POS_3^{2-}, PO_3NH^{2-}, PO_3NHR^- [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], NO_2, $CONH_2$, COOR [R=alkyl (C1-22), acyl (C1-22), substituted (alkyl, amino, alkoxy, halogen, or the like) or unsubstituted aryl]; B represents a natural base or a modified base or H; Y represents rest of the enzymatic nucleic acid; and R1 represents H, O-alkyl, C-alkyl, halo, NHR [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], or OCH_2SCH_3 (methylthiomethyl). The 5'-modified sugar synthesis is as described by Moffatt, in *Nucleoside Analogues:Chemistry, Biology and Medical Applications*, Walker, DeClercq, and Eckstein, Eds,; Plenum Press:New York, 1979, pp 71 (incorporated by reference herein).

Another preferred embodiment of the invention features enzymatic nucleic acid molecules having a 5'-cap, wherein said cap is selected from but not limited to, a group comprising, 4',5'-methylene nucleotide; 1-(β-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl phosphate; 12-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; α-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moeity; 5'-5'-inverted abasic moeity; 5'-phosphoramidate; 5'-phosphorothioate; 1, 4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moeities (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

In a third aspect, the invention features enzymatic nucleic acids with 3'-end modifications (3'-cap) having the formula:



wherein, X represents 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; α-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moeity; 3'-3'-inverted abasic moeity; 3'-2'-inverted nucleotide moeity; 3'-2'-inverted abasic moeity; 1,4-butanediol; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; or bridging or nonbridging methylphosphonate moeity; B represents a natural base or a modified base or H; Y represents rest of the enzymatic nucleic acid; and R1 represents H, O-alkyl, *C*-alkyl, halo, NHR [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], or OCH_2SCH_3 (methylthiomethyl).

In yet another preferred embodiment the invention features enzymatic nucleic acid molecules having a 3'-cap, wherein said cap is selected from but not limited to, a group comprising, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; α-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moeity; 3'-3'-inverted abasic moeity; 3'-2'-inverted nucleotide moeity; 3'-2'-inverted abasic moeity; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or nonbridging methylphosphonate moeity (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

In a fourth aspect, the invention features enzymatic nucleic acids with both 5'-cap and a 3'-cap which may be same or different.

The term "nucleotide" is used as recognized in the art to include natural bases, and modified bases well known in the art. Such bases are generally located at the 1' position of a sugar moiety. Nucleotide generally comprise a base, sugar and a phosphate group. The nucleotide can be unmodified or modified at the sugar, phosphate and/or base moeity. The term "abasic" or "abasic nucleotide" as used herein encompasses sugar moieties lacking a base or having other chemical groups in place of base at the 1' position.

By the phrase "enzymatic nucleic acid" is meant a catalytic modified-nucleotide-containing nucleic acid molecule that has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity that specifically cleaves RNA or DNA in that target. That is, the enzymatic nucleic acid is able to intramolecularly or intermolecularly cleave RNA or DNA and thereby inactivate a target RNA or DNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA or DNA to allow the cleavage to occur. 100% Complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention. The nucleic acids may be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, minizyme, leadzyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity.

There are several examples of modified bases as it relates to nucleic acids, is well known in the art and has recently been summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into enzymatic nucleic acids without significantly effecting their catalytic activity include, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyluracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-

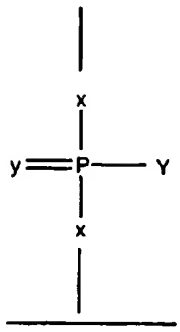
methyluridine); Guanosine or adenosine residues may be replaced by diaminopurine residues in either the core or stems.

There are several examples in the art describing sugar modifications that can be introduced into enzymatic nucleic acid molecules without significantly effecting catalysis and significantly enhancing their nuclease stability and efficacy. Sugar modification of enzymatic nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, *International Publication* PCT No. WO 92/07065; Perrault *et al. Nature* 1990, *344*, 565-568; Pieken *et al. Science* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *US Patent* No. 5,334,711 and Beigelman *et al.*, 1995 *J. Biol. Chem.* 270, 25702). Such publications describe the location of incorporation of modifications and the like, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein.

The 5'-cap and/or 3'-cap derivatives of this invention provide enhanced activity and stability to the enzymatic nucleic acids containing them.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with other RNA sequence by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

By "bridging" and "nonbridging" are meant to indicate the relative positions of oxygen atom involved in the formation of standard phosphodiester linkage in a nucleic acid. These backbone oxygen atoms can be readily modified to impart resistance against nuclease digestion. The terms are further defined as follows:



Wherein "x "is bridging oxygen and 'y' is nonbridging oxygen.

In preferred embodiments of this invention, the enzymatic nucleic acid molecule is formed in a hammerhead or hairpin motif, but may also be formed in the motif of a hepatitis delta virus (HDV), group I intron, RNaseP RNA (in association with an RNA guide sequence) or *Neurospora* VS RNA. Examples of such hammerhead motifs are described by Rossi *et al.*, 1992, *Aids Research and Human Retroviruses* 8, 183, of hairpin motifs by Hampel *et al.*, EP0360257, Hampel and Tritz, 1989 *Biochemistry* 28, 4929, and Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299, and an example of the hepatitis delta virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16; of the RNaseP motif by Guerrier-Takada *et al.*, 1983 *Cell* 35, 849 and Forster and Altman, 1990 *Science* 249, 783, *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Guo and Collins, 1995 *EMBO J.* 14, 368) and of the Group I Intron by Cech *et al.*, U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

The invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the RNA of a

desired target. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of a target such that specific treatment of a disease or condition can be provided with a single enzymatic nucleic acid. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required. In the preferred hammerhead motif the small size (less than 60 nucleotides, preferably between 30-40 nucleotides in length) of the molecule allows the cost of treatment to be reduced compared to other ribozyme motifs.

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small enzymatic nucleic acid motifs (*e.g.*, of the hammerhead structure) are used for exogenous delivery. The simple structure of these molecules increases the ability of the enzymatic nucleic acid to invade targeted regions of the mRNA structure. Unlike the situation when the hammerhead structure is included within longer transcripts, there are no non-enzymatic nucleic acid flanking sequences to interfere with correct folding of the enzymatic nucleic acid structure or with complementary regions.

Therapeutic ribozymes must remain stable within cells until translation of the target mRNA has been inhibited long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Clearly, ribozymes must be resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of RNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; incorporated by reference herein) have expanded the ability to modify ribozymes to enhance their nuclease stability. The majority of this work has been performed using hammerhead ribozymes (reviewed in Usman and McSwiggen, 1995 *supra*) and can be readily extended to other ribozyme motifs.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawing will first briefly be described.

Drawing:

Figure 1 is a diagrammatic representation of the hammerhead ribozyme domain known in the art. Stem II can be ≥ 2 base-pair long. Each N is independently any base or non-nucleotide as used herein.

Figure 2a is a diagrammatic representation of the hammerhead ribozyme domain known in the art; Figure 2b is a diagrammatic representation of the hammerhead ribozyme as divided by Uhlenbeck (1987, *Nature*, 327, 596-600) into a substrate and enzyme portion; Figure 2c is a similar diagram showing the hammerhead divided by Haseloff and Gerlach (1988, *Nature*, 334, 585-591) into two portions; and Figure 2d is a similar diagram showing the hammerhead divided by Jeffries and Symons (1989, *Nucl. Acids. Res.*, 17, 1371-1371) into two portions.

Figure 3 is a diagrammatic representation of the general structure of a hairpin ribozyme. Helix 2 (H2) is provided with a least 4 base pairs (*i.e.*, n is 1, 2, 3 or 4) and helix 5 can be optionally provided of length 2 or more bases (preferably 3 - 20 bases, *i.e.*, m is from 1 - 20 or more). Helix 2 and helix 5 may be covalently linked by one or more bases (*i.e.*, r is ≥ 1 base). Helix 1, 4 or 5 may also be extended by 2 or more base pairs (*e.g.*, 4 - 20 base pairs) to stabilize the ribozyme structure, and preferably is a protein binding site. In each instance, each N and N' independently is any normal or modified base and each dash represents a potential base-pairing interaction. These nucleotides may be modified at the sugar, base or phosphate. Complete base-pairing is not required in the helices, but is preferred. Helix 1 and 4 can be of any size (*i.e.*, o and p is each independently from 0 to any number, *e.g.*, 20) as long as some base-pairing is maintained. Essential bases are shown as specific bases in the structure, but those in the art will recognize that one or more may be modified chemically (abasic, base, sugar and/or phosphate modifications) or replaced with another base without significant effect. Helix 4 can be formed from two separate molecules, *i.e.*, without a connecting loop. The connecting loop when present may be a ribonucleotide with or without modifications to its base, sugar or phosphate. "q" is ≥ 2 bases. The

connecting loop can also be replaced with a non-nucleotide linker molecule. H refers to bases A, U, or C. Y refers to pyrimidine bases. "_____" refers to a covalent bond.

Figure 4 is a representation of the general structure of the hepatitis delta virus ribozyme domain known in the art. In each instance, each N and N' independently is any normal or modified base and each dash represents a potential base-pairing interaction. These nucleotides may be modified at the sugar, base or phosphate.

Figure 5 is a representation of the general structure of the self-cleaving VS RNA ribozyme domain.

Figure 6 is a diagrammatic representation of a scheme involved in the synthesis of 2,2'-anhydro-1(β-D-arabinofuranosyl) uracil (2).

Figure 7 is a diagrammatic representation of a scheme involved in the synthesis of 2'-*O*-methyl uridine (3) by the method of this invention.

Figure 8 is a diagrammatic representation of a scheme involved in the synthesis of 2'-*O*-methyl cytidine (5) by the method of this invention.

Figure 9 is a diagrammatic representation of a scheme involved in the synthesis of 3'-*O*-methyl pyrimidine nucleosides.

Figure 10 is a diagrammatic representation of a scheme involved in the synthesis of 5'-*O*-methyl pyrimidine nucleosides.

Figure 11 shows NMR profile of 2'-*O*-methyl nucleosides. A) 2'-*O*-methyl Uridine nucleoside. B) 2'-*O*-methyl cytidine nucleoside.

Figure 12 is a diagrammatic representation of a scheme for the synthesis of 2'-*O*-methyl adenosine nucleoside.

Figure 13 is a diagrammatic representation of a scheme for the synthesis of 2'-*O*-methyl adenosine and guanosine nucleosides.

Figure 14 is a diagrammatic representation of a scheme for the synthesis of 2'-*O*-methyl guanosine nucleoside.

Figure 15 is a diagrammatic representation of a scheme for the synthesis of methoxy nucleosides via N1-Benzyl guanosine route.

Figure 16 shows a strategy used in synthesizing a hammerhead ribozyme from two halves. X and Y represent reactive moieties that can undergo a chemical reaction to form a covalent bond (represented by the solid curved line).

Figure 17 shows various non-limiting examples of reactive moieties that can be placed in the nascent loop region to form a covalent bond to provide a full-length ribozyme. CH_2 can be any linking chain as described above including groups such as methylenes, ether, ethylene glycol, thioethers, double bonds, aromatic groups and others; each n independently is an integer from 0 to 10 inclusive and may be the same or different; each R independently is a proton or an alkyl, alkenyl and other functional groups or conjugates such as peptides, steroids, hoemones, lipids, nucleic acid sequences and others that provides nuclease resistance, improved cell association, improved cellular uptake or interacellular localization.

Figure 18 shows non-limiting examples of covalent bonds that can be formed to provide the full length ribozyme. The morpholino group arises from reductive reaction of a dialdehyde, which arises from oxidative cleavage of a ribose at the 3'-end of one half ribozyme with an amine at the 5'-end of the half ribozyme. The amide bond is produced when an acid at the 3'-end of one half ribozyme is coupled to an amine at the 5'-end of the other half ribozyme.

Figure 19 shows non-limiting examples of three ribozymes that were synthesized from coupling reactions of two halves. All three were targeted to the 575 site of c-*myb* RNA. Rz 1 was formed from the reaction of two thiols to provide the disulfide linked ribozyme. Rz 2 and Rz 3 were formed using the morpholino reaction. Rz 2 contains a five atom spacer linking the terminal amine to the 5'-end of the half ribozyme. Rz 3 contains a six carbon spacer linking the terminal amine to the 5'-end of the half ribozyme.

Figure 20 shows comparative cleavage activity of half ribozymes, containing five and six base pair stem II regions, that are not covalently linked

vs a full length ribozyme. Assays were carried out under ribozyme excess conditions.

Figure 21 shows comparative cleavage activity of half ribozymes, containing seven and eight base pair stem II regions, that are not covalently linked vs a full length ribozyme. Assays were carried out under ribozyme excess conditions.

Figure 22 shows comparative cleavage assay of Rz 1, 2 and 3 (see Figure 19) formed from crosslinking reactions vs a full length ribozyme control. Assays were carried out under ribozyme excess conditions.

Figure 23 is a diagrammatic representation of c-*myb* site 575 hammerhead half-ribozymes and substrate RNA. The arrow indicates the site of cleavage.

Figure 24 A) shows the synthesis of acyclic 2', 3'-dialdehyde-5'-half riboyme (**4**) and 3'-phosphoryl-5'-half-ribozyme (**5**) from 3'-uridilyl-5'-half-ribozyme (**1**). B) shows anion exchange HPLC analysis of reaction of 1 with $NaIO_4$ at room temperature. C) shows the HPLC profile of ammonium acetate desalted compound **4**.

Figure 25 is a schematic representation of synthesis of morpholino-linked ribozymes.

Figure 26 shows an anion-exchange HPLC analysis of the reductive alkylation of compunds **2** and **4**.

Figure 27 is a diagrammatic representation of a hammerhead ribozyme-substrate complex. The ribozyme is targeted against site 575 within c-*myb* RNA. Lowercase alphabets indicate 2'-*O*-methyl substitution; uppercase alphabets indicate ribonucleotides; Arrow incates the site of RNA cleavage; $\underline{u}_4$ and $\underline{u}_7$, represent modification with 2'-amino group; and , represent 5'- and 3'- caps which may be same or different.

Figure 28 A) is a general formula for 5'-end modifications. B) chemical structures of a few of the 5'-end modifications. C) diagrammatic representation of a 5'-5'-inverted abasic moiety.

Figure 29 A) diagrammatic representation of 5'-phosphoramidate and 5'-phosphorothioate linkages; B) a synthesis scheme for 5'-amino-5'-deoxy-2'-*O*-methyl uridine and guanosine phosphoramidites; C) a synthesis scheme for 5'-amino-5'-deoxy-2'-*O*-methyl adenosine phosphoramidites; D) a synthesis scheme for 5'-deoxy-5'-mercapto-2'-*O*-methyl uridine and cytidine phosphoramidites.

Figure 30 shows ribozyme-mediated inhibition of smooth muscle cell proliferation. The hammerhead (HH) ribozymes, targeted to site 575 within c-*myb* RNA, as shown in Figure 28, were chemically modified such that the ribozyme consists of ribose residues at five positions; U4 and U7 positions contain 2'-NH_2 modifications, the remaining nucleotide positions contain 2'-*O*-methyl substitutions. Additionally, the 5'-end of the ribozyme contains 5'-amino modification and the 3' end of the ribozyme contains a 3'-3' linked inverted abasic deoxyribose (designated as 3'-iH). Inactive ribozyme (5'-amino Inactive RZ) with G5 to U and A14 to U substitution was synthesized and used as a negative control.

Figure 31 shows ribozyme-mediated inhibition of smooth muscle cell proliferation. The hammerhead (HH) ribozymes, targeted to site 575 within c-*myb* RNA, as shown in Figure 28, were chemically modified such that the ribozyme consists of ribose residues at five positions; U4 position contains 2'-*C*-allyl modification, the remaining nucleotide positions contain 2'-*O*-methyl substitutions. Additionally, the 5'-end of the ribozyme contains amino modification and the 3' end of the ribozyme contains a 3'-3' linked inverted abasic deoxyribose (designated as 3'-iH). Inactive ribozyme (5'-amino Inactive RZ) with G5 to U and A14 to U substitution were synthesized and used as negative control.

Figure 32 A) chemical structures of a few of the 3'-end modifications. B) diagrammatic representation of a few 3'-end mofication linkages.

Figure 33 is a synthesis scheme for phosphorodithioate linkages.

Figure 34 is a synthesis scheme for 3'-2'-inverted nucleoside or an abasic nucleoside linkages. Compound 2 can be reacted with compound 3 to yield either a 3'-2'-inverted nucleotide linkage as shown in Figure 32B, *infra*, or

a 3'-2'-inverted abasic ribose, deoxyribose or variations thereof (see Figure 32B).

Figure 35 is a synthesis scheme for carbocyclic nucleoside phosphoramidite.

Figure 36 is a synthesis scheme for alpha nucleoside phosphoramidite.

Figure 37 is a synthesis scheme for 1-(β-D-erythrofuranosyl) nucleoside phosphoramidite.

Figure 38 is a synthesis scheme for inverted deoxyabasic 5'-*O*-succinate and 5'-*O*-phosphoramidite.

Figure 39 is a graphical representation of RNA cleavage reaction catalyzed by hammerhead ribozymes containing either one or two 5'-terminal phosphorodithioate modifications. Ribozyme .4654/1 5'-dithio, represents a hammerhead ribozyme targeted to *c-myb* site 575 as shown in Figure 28, and were chemically modified such that the ribozyme consists of ribose residues at five positions; U4 position contains 2'-*C*-allyl modification, the remaining nucleotide positions contain 2'-*O*-methyl substitutions. Additionally, the 5'-end of the ribozyme contains one phosphorodithioate modification and the 3' end of the ribozyme contains a 3'-3' linked inverted abasic deoxyribose (designated as 3'-iH). Ribozyme .4657/2 5'-dithio, represents a hammerhead ribozyme targeted to *c-myb* site 575 as shown in Figure 28, and were chemically modified such that the ribozyme consists of ribose residues at five positions; U4 position contains 2'-*C*-allyl modification, the remaining nucleotide positions contain 2'-*O*-methyl substitutions. Additionally, the 5'-end of the ribozyme contains two phosphorodithioate modification and the 3' end of the ribozyme contains a 3'-3' linked inverted abasic deoxyribose (designated as 3'-iH).

Examples

The following are non-limiting examples showing the synthesis of methoxy nucleosides, synthesis and activity of enzymatic nucleic acids

containing 5'- and/or 3'-cap modifications and the synthesis of monomer phosphoramidites.

Example 1: Synthesis of 2,2'-anhydro-1(β-D-arabinofuranosyl) uracil (2)

2,2'-anhydro-1(β-D-arabinofuranosyl) uracil (2) can either be purchased from Sigma Chemicals or can be synthesized using the scheme described by Verheyden *et al.*, 1971 (*J. Org. Chem.* 36, 250). Briefly, to an oven baked 1L 3-neck round bottom flask equipped with mechanical stirrer, reflux condenser, and positive pressure of argon, 200g (0.819 mol) of uridine (1) was added. The reaction was carried out in the presence of diphenylcarbonate (191.2 g, 0.9 mol), and DMF (300 ml). The resulting light yellow suspension was heated to 90°C at which time sodium bicarbonate (2.0 g) was added. The reaction mixture was then heated to 110°C for two hours during which time CO_2 evolved. Over this two hour period, the reaction mixture transformed from a slurry to a homogeneous solution and back to a slurry. Upon cooling to -10°C, the reaction mixture was filtered and the filter bed washed with ethanol and cold methanol. The filter bed was then suspended in methanol (500 ml) and heated to reflux for three hours. After cooling to -10°C, the reaction mixture was filtered. The filter bed was washed with cold methanol and dried to retrieve 2 as an off white solid (140g; 76%).

Example 2: Synthesis of 2'-*O*-methyl uridine (3)

To an oven baked stainless steel bomb (300 ml), equipped with magnetic stirrer and purged with argon, 40 ml anhydrous methanol was added followed by the addition of 2,2'-anhydro-1-(β-D-arabinofuranosyl)uracil 2 (1.0 g, 4.42 mmol). To the resulting slurry, 5.0 ml trimethylborate (44.2 mmol) was added followed by the addition of boron trifluoride-methanol (50%) (1.5 ml, 8.84 mmol). The bomb was then sealed and heated in an oil bath at 130°C for 18 hours. Upon cooling, the resulting clear, slightly colored reaction mixture was evaporated *in vacuo* to yield a dark foam. The crude foam was dissolved in minimal methanol/dichloromethane and applied to a flash silica gel column. A gradient of 10-30% EtOH in dichloromethane afforded 3 as a white foam (1.05 g, 92%).

Alternately, to an oven baked stainless steel bomb (920 ml), equipped with magnetic stirrer and purged with argon, 200 ml anhydrous methanol was added followed by the addition of 2 (50 g, 0.221 mol). Trimethylborate (400 ml, 3.54 mol) was added to the resulting slurry and the bomb was sealed. The bomb was then heated in an oil bath at 130°C for 38 hours. Upon cooling, the resulting clear, slightly colored reaction mixture was evaporated *in vacuo* to afford an off white foam. Crystallization of the crude product from (methanol/ethyl acetate) gave pure 3 (56.8 g, 100%).

The identity and purity of the synthesized compound was confirmed by standard NMR analysis (Figure 11A). Following is the result of NMR analysis: ^{1}H NMR DMSO: 11.33 (exch. s, 1H, NH), 7.92 (d, $J_{6,5}$=8.2, 1H, H6), 5.85 (d, $J_{1',2'}$=5.2, 1H, H1'), 5.65 (d, $J_{5,6}$=8.2, 1H, H5), 5.13 (exch. m, 2H, 5'OH, 3'OH), 4.10 (t, $J_{3',2'}$=4.9, $J_{3',4'}$=4.6, 1H, H3'), 3.85 (m, 1H, H4'), 3.62 (dd, $J_{5',4'}$=3.0, $J_{5',5''}$=12.1, 1H, H5'), 3.54 (dd, $J_{5'',4'}$=3.1, $J_{5'',5'}$=12.1, 1H, H5''), 3.35 (s, 3H, OCH_3). The peak corresponding to the 2'-*O*-methyl is indicated in the figure 11A.

Example 3: 2'-*O*-methyl cytidine (5)

To an oven baked stainless steel bomb (300 mL), equipped with magnetic stirrer and purged with argon, 50 ml anhydrous methanol was added followed by the addition of commercially available 1g 2,2'-Anhydro-1-(β-D-arabinofuranosyl)cytosine•acetate (Aldrich) 4 (3.5 mmol). To the resulting slurry, 8 ml Trimethylborate (70 mmol) was added in the presence or absence of boron trifluoride-methanol (50%) (1.5 ml, 8.84 mmol). The bomb was sealed and then heated in an oil bath at 130°C for 38-48 hours. Upon cooling, the resulting clear, slightly colored reaction mixture was evaporated *in vacuo* to afford an off white foam. After drying *in vacuo*, the crude foam was dissolved in anhydrous DMF (50 ml) and acetic anhydride (0.36 ml, 3.85 mmol) which was added drop-wise to the reaction mixture. The resulting clear, light yellow solution was stirred overnight at room temperature. The reaction mixture was evaporated *in vacuo*. Crystallization of the crude product from (methanol/ethyl acetate) gave a pure compound 5 (0.94 g, 90%).

The identity and purity of the synthesized compound was confirmed by standard NMR analysis (Figure 11B). Following is the result of NMR analysis:

^{1}H NMR DMSO: 10.89 (exch. s, 1H, NH), 8.46 (d, $J_{6,5}$=7.4, 1H, H6), 7.18 (d, $J_{5,6}$=7.4, 1H, H5), 5.83 (d, $J_{1',2'}$=2.5, 1H, H1'), 5.18 (exch. t, $J_{OH,5'}$=4.6, $J_{OH,5''}$=4.9, 1H, 5'OH), 5.08 (exch. d, $J_{OH,3'}$=6.7, 1H, 3'OH), 4.04 (t, $J_{3',2'}$=4.9, $J_{3',4'}$=6.8, 1H, H3'), 3.88 (m, 1H, H4'), 3.75 (dd, $J_{5',4'}$=2.3, $J_{5',5''}$=12.2, 1H, H5'), 3.59 (dd, $J_{5'',4'}$=2.5, $J_{5'',5'}$=12.2, 1H, H5''), 3.45 (s, 3H, OCH_3), 2.10 (s, 3H, CH_3).

2'-*O*-methyl nucleosides of the present invention can be readily converted into phosphoramidites using standard procedures and phosphoramidites can be readily incorporated into oligonucleotides, such as RNA, using standard procedures described in Sproat & Gait, 1984 in *Oligonucleotide Synthesis: A Practical Approach,* ed. Gait, M. J. (IRL, Oxford), pp 83-115; Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684.

Example 4: Synthesis of 3'-*O*-methyl Pyrimidine Nucleoside

Referring to Figure 9, the treatment of 2,3'-anhydro-1-(β-D-arabinofuranosyl)primidine 6 (Aldrich) with anhydrous methanol and trimethylborate, in the presence or absence of boron trifluoride-methanol, in an oven baked stainless steel bomb purged with argon, followed by heating in an oil bath at 130°C for 18-48 hours, as described above, will yield 3'-*O*-methyl pyrimidine nucleoside 7.

Example 5: Synthesis of 5'-*O*-methyl Pyrimidine Nucleoside

Referring to Figure 10, the treatment of 2,5'--anhydro-1-(β-D-arabinofuranosyl)primidine 8 (Aldrich) with anhydrous methanol and trimethylborate, in the presence or absence of boron trifluoride-methanol, in an oven baked stainless steel bomb which is purged with argon, followed by heating in an oil bath at 130°C for 18-48 hours, as described above, will yield 5'-*O*-methyl pyrimidine nucleoside 9.

Example 6: Synthesis of 2'-O-Me -Adenosine via transglycosilation (**Scheme 1; Figure 12)**

In 1982, Imbach *et al.*, (*J. Org. Chem.* 1982, 47, 202) demonstrated utilization of 2'-*O*-methyl-1,3,5-tri-*O*-benzoyl-α-D-ribofuranose in the stereospecific synthesis of 2'-*O*-methyl pyrimidine-β-D-ribonucleoside by the

glycosilation of silylated bases, using Lewis acid as a catalyst. This procedure was optimized for large scale preparation of 2'-*O*-methylpyrimidine ribonucleosides by Ross *et al.*, (*J. Hetercyclic Chem.* 31,765 1994). This procedure required methylation of 1,3,5-tri-O-benzoyl -α -D-ribofuranose with a large excess of potentially explosive diazomethane.

As an alternate, Applicant describes transglycosilation of suitably protected 2'-*O*-Me-pyrimidine ribonucleosides obtained through $B(OMe)_3$ mediated opening of 2,2'-anhydronycleosides. Transglycosylation reaction proceeds usually with high β-selectivity, if the carbohydrate donor contains a 2'-*O*-acyl group capable of stabilising the postulated C-1 carboxonium ion for exclusive β- attack by the incoming silylated base. The stereochemical outcome of transglycosilation reaction with carbohydrate donor, without the participating group at 2'-position (as 2'-*O*-Methyl) usually results in a mixture of α, and β nucleosides, as documented for the synthesis of 2'-N_3 purine ribonucleoside (Imazawa and Eckstein, 1979, *J.Org. Chem.* 44, 2039-41).

Applicant has investigated transglycosilation of 5',3'-di-*O*-acetyl-2'-*O*-Methyl uridine, obtained by the acylation of 2'-*O*-Methyl uridine. The transglycosilation of 5',3'-di-*O*-acetyl-2'-*O*-Methyl uridine with 3 eq of N^6-benzoylaminopurine and 3 eq TMStriflate in CH_3CN at 75°C for 16 hour resulted in unseparable mixture of α and β isomers of *N^6-Benzoyl-5',3'-di-O-acetyl-2'-O-methyl adenosine* in ~60% yield and 1:1 ratio. Since the nature of aglycone can also influence product distribution in transglycosilation reaction (Azuma and Isono, 1977, *Chem. Pharm. Bull.* 25, 3347-53), we decided to try N^4-acetyl-5',3'-di-*O*-acetyl-2'-*O*-methyl cytidine (2) as a carbohydrate donor. Suprisingly, utilization of this donor under the same conditions as described above for 2'-*O*-Methyl uridine derivative resulted in the exclusive formation of β anomer of *N^6-Benzoyl-5',3'-di-O-acetyl-2'-O-methyl adenosine* (3) in 50% yield. When N^6-phenoxyacetylaminopurine was used instead of *N^6-Benzoyl*-adenine under the same conditions described above, extensive decomposition of initially formed adenosine derivative was observed and target nucleoside was not isolated.

Example 7: Synthesis of 2'-*O*-Methyl-Guanosine and Adenosine from 2-amino-6-chloropurine riboside (**Scheme 2; Figure 13**).

High regioselectivity in methylation of 6-Cl-guanosine by diazomethane was reported by Robins' laboratory in 1966 (Khawaia and Robins, *J. Am. Chem. Soc.* 1966, 88 ,3640-43).. Despite the exclusive methylation of 2'-OH, the 2'-*O*-Methyl-6-Cl-guanosine was not isolated. Subsequent transformation of this key intermediate resulted in the preparation of 2'-O-methyl-guanosine in 30% yield. Moreover, the use of diazomethane as a methylation agent is not practical for large scale preparations. It was therefore reasonable to investigate other methylation reagents.

Suprisingly, Applicant has found that the methylation of 2-amino-6-chloropurine riboside with a small excees of NaH/MeI reagent in CH_2Cl_2 at -20° C resulted in 2'- *O*- Me-6-Cl-guanosine in 65 % yield along with the formation of 2',3' bis-*O*-Me derivative in 15 % yield; no 3'- methylation was observed under these conditions. Several procedures were tested for the transformation of intermediate (**5**) into 2'-*O*-Methyl-guanosine (**9**). Applicant found that, the best result was obtained when intermediate (**5**) was treated with 1,4-diazabicyclo[2.2.2]octane (1 equiv.) and water (30 mL) at 90°C for 45 minutes, followed by hydrolysis with 2M NaOH at pH 12 (Figure 13). The desired 2'-*O*-Methyl-guanosine was obtained in 65 % yield.

The surprisingly high regio-selectivity observed in the methylation of 2-amino-6-chloropurine riboside facilitates large scale synthesis of 2'- *O*- Me-6-Cl-guanosine, which can serve as a key intermediate in the preparation of not only 2'-*O*-Methyl-guanosine, but also 2'-*O*-Methyl-adenosine. This latter transformation was achieved through radical deamination (Nair and Richardson, *Synthesis,* 1982 670-672) of 3',5'-di-*O*-Acetyl-2'-*O*-Methyl-6-Chloro-2-aminopurine riboside (**6**) which yields 3',5'-di-*O*-Acetyl-2'-*O*-Methyl-6-Chloropurine (**7**) in 72 % yield starting from **5**. Subsequent amination of (**7**) with methanolic ammonia at 125°C for 4 hours yields 2'-*O*-Methyl-adenosine (**8**) in 80% yield.

Example 8: Synthesis of N^2-acyl(Isobutyryl and isopropylphenoxyacetyl) -2'-*O*-methylguanosine from 2,6-Diamino-β-D-ribofuranosylpurine (Scheme 3; Figure 14)

It has been reported that the diazomethane methylation of *2,6-Diamino-β-D-ribofuranosylpurine* in the presence of $SnCl_2$ x 2 H_2O provided a 1:1 mixture of 2'- and 3'-*O*-methylated derivatives in a quantitative yield. These compounds can be separated on Dowex 1 OH^- column and deaminated to the corresponding Guanosine derivatives with Adenosine Deaminase (Robins *et al., Can. J. Chem.* 1981, 59, 3360). It is also known in the literature that direct methylation of guanosine usually resulted in preferential methylation of N_1 and/or N_7 positions of the base. In *2,6-Diamino-β-D-ribofuranosylpurine,* the acidic amide function C6-N1- is replaced by an basic amidine function, therefore one would expect that such a replacement should reduce the extent of methylation at N1 under basic conditions (i.e. NaH/MeI). In order to increase the regioselectivity, Applicant used 5',3'-*O-tetraisopropyldisiloxane-1,3-diyl* protection which has been reported to be relatively stable under NaH/MeI methylation conditions (Parmentier *et al.,* 1994, *Tetrahedron,* 50, 5361-68).

The 5',3'-*O-tetraisopropyldisiloxane-1,3-diyl* protection was introduced by standard procedure, utilization of ethylacetate-water extraction allowed isolation of pure protected derivative (11) in 90% yield in crystalline form due to low solubility in above system.

Applicant has investigated several methylation procedures for the NaH/MeI system, including, varying the amount and the type of solvent and equivalents of NaH and MeI. Best results were obtained when methylation was performed at O°C in DMF with 1.5 eq of NaH and 3 eq. of MeI. If the reaction is performed at higher temperatures or in a more concentrated solution, extensive hyper-methylation occured. Utilization of lower amounts of MeI required longer reaction time and resulted in the opening of cyclic silyl group with the simultaneous methylation of both 2' and 3' hydroxyl groups. The 2'-*O*-Methyl derivative 12 can then be isolated in 90% yield by crystallization and without any column chromatography (Figure 14).

In order to synthesize *N^2-acyl -2'-O-methylguanosine* derivatives **14** and **15**, Applicant tested selective acylation of N^2 amino group of intermediate **12** with subsequent chemical deamination of N^2 amino group. Two factors are critical for the success of this approach: the degree of selectivity in acylation of N^2 amino group vs N^6 amino group in intermediate **12** and the stability of N^2 protection under acidic conditions of deamination (Davoll *et al., J Am. Chem. Soc.* 1951, 73, 1650). Applicant has found that when the acylation of diamonopurine **12** is performed at -10°C with 1.1 eq of acyl chloride, exclusive acylation of N^2 amino group in **12** occured (Figure 14). For example, the N^2-isobutyryl intermediate **13** was isolated in 97% yield. The structure of **13** was confirmed by NMR data and by deamination to *N^2-isobutyryl-2'-O-methylguanosine* **14** with $NaNO_2/CH_3COOH$ followed by desilylation with TEA•3HF. Applicant also observed that during deamination with $NaNO_2/CH_3COOH$, 3',5'-*O*-cyclic silyl group opened, presumably at the 5'-position. This intermediate was not isolated but desilylated directly with TEA•3HF. It is worth noting that N^2-isobutyryl group was completely stable during deamination. The presence of hydrophobic silyl group in the intermediate allowed easy separation by extraction from excess of inorganic salts which made possible the subsequent desilylation without isolation.

High yields obtained in all the steps of transformation from **12** to **14** prompted us to combine these reactions in a "one pot" procedure without the isolation of intermediate **13**. Selective acylation of **12** with isobutyryl chloride followed by deamination with $NaNO_2/CH_3COOH$ and desylilation with TEA•3HF resulted in *N^2-isobutyryl-2'-O-methylguanosine* **14** in 88% yield.

We also applied this procedure for the synthesis of *N^2-isopropylphenoxyacetyl-2'-O-methylguanosine* on a 50 g scale. The *N^2-isopropylphenoxyacetyl* protection was also stable under acidic deazotation conditions, athough a minor loss of this group was observed resulting in a 83% overall yield starting from **12**.

Example 9: Synthesis of *2'-O-methylguanosine* and 2'-O-Methyl-adenosine via metal-directed methylation **(Scheme 4; Figure 15 and Table IX)**

It has been demonstrated that metal acetylacetonates can direct methylation of ribonucleosides with trimethylsulfonium hydroxide, mostly at the 2' and 3' hydroxyl groups of Uridine, Cytidine and Adenosine (Yamauchi *et al., J. Org Chem.* 1980, 45, 3865-68). Application of this procedure to the synthesis of Guanosine derivatives resulted in the isolation of 6 compounds with methylation in the base and the ribose moeties. When N^1-methyl-guanosine was subjected to the same methylation conditions 1,2' and 1,3' di-N-*O* methyl guanosines were isolated in 82% yield.

Applicant investigated procedures for metal-directed methylation of N^1-protected Guanosine with trimethylsulfonium hydroxide to optimize the ratio of 2' : 3' methylated products with subsequent separation and debloking to obtain 2'-*O*-Methyl guanosine.

The protection of N^1 in guanosine was achieved using N^1 benzylation with N,N-dimethylformamide dibenzyl acetal (Philips and Horwitz *J.Org. Chem.* 1975, 40,1856). Applicant observed that complete cleavage of 2',3'-orthoamide required more drastic conditions than previously reported (2N NaOH vs $MeOH/NH_3$) (Figure 15). The target compound was isolated in 80% yield after crystallization.

Several metal acetylacetonates were tested in methylation reaction (See Table I). Whereas Cu^{2+} acetylacetonate mediated methylation produced 1:1 ratio of 2'- and 3'-*O*-methylated products; Mg^{2+} and Ag^{+} directed methylation changed the ratio to 9:1. With Ag^{+} the overall conversion was higer than with Mg^{2+} resulting in a 70% isolated yield of 2'-*O*-Me-N_1-Bzl-guanosine (Figure 15; **17**). The separation of 2'-*O*-and 3'-*O*-Me-N_1-Bzl-guanosine derivative was achieved on a preparative scale on Waters Delta-Pak ODS 50mm x 300mm HPLC column. Removal of N_1-Bzl protection with Na^{+} naphtalene provided 2'-*O*- Me-Guanosine in 90% yield.

Applicant investigated 12 different acetylacetonates in the direct methylation of Adenosine (Table II). Whereas Fe and Cu acetylacetonates provided 1:1 and 2:1 ration of 2' and 3' isomers as reported, Applicant

discovered that Ag^{+} and Sr^{2+} allows equilibrium to shift towards 2'-isomer, providing 4:1 and 8:1 ratio. This allows the isolation of 2'-*O*-Me adenosine in 75-80% yield.

Experimental Procedures for the synthesis of 2'-O-methyl adenosine and guanosine nucleosides

NMR spectra were recorded on a Varian Gemini 400 spectrometer operating at 400.075 MHz for proton and 161.947 MHz for phosphorus. Chemical shifts in ppm refer to TMS and H_3PO_4, respectively. Analytical thin-layer chromatography (TLC) was performed with Whatman MK6F silica gel 60 Å F_{254} plates and column chromatography using Merck 0.040-0.063 mm Silica gel 60.

N^6-Benzoyl-5',3'-di-O-acetyl-2'-O-methyl adenosine (3) :

To a solution of N^4-acetyl-2'-O-methyl cytidine(1) (1.87 g, 6.25 mmol) stirring at RT under argon in DMF/pyridine (20 ml, 20 ml) was added acetic anhydride (1.76 ml, 18.75 mmol) via syringe. The reaction mixture was stirred at RT for 18 hours then quenched with EtOH (2 ml). The reaction mixture was evaporated to dryness *in vacuo* and partitioned between dichloromethane and sat. $NaHCO_3$. The aqueous layer was back extracted with additional dichloromethane and the combined organics dried over Na_2SO_4. After filtration, the filtrate was evaporated *in vacuo* to afford a white foam.

A solution of N^6-benzoylaminopurine (Lancaster) (1.23 g, 5.16 mmol) stirring in anhydrous acetonitrile under an argon atmosphere was treated with BSA (3.82 ml, 15.48 mmol) at reflux for 3 hours. Upon cooling, a solution of N^4-acetyl-5',3'-di-O-acetyl-2'-O-methyl cytidine **(2)** (see above) (0.66 g, 1.72 mmol) in 20 ml anh. acetonitrile was added to the reaction mixture followed by TMStriflate (1.03 ml, 5.16 mmol). The reaction mixture was then heated to 75°C for 16 hours while stirring under positive pressure argon. Upon cooling, an additional 1.03 ml (5.16 mmol) of TMStriflate was added, and the reaction heated to 75°C for an additional 20 hours. Once cool, the reaction mixture was diluted with two volumes of dichloromethane and washed with sat. $NaHCO_3$. The organic layer was then dried over Na_2SO_4 and evaporated *in vacuo*. Flash chromatography employing a gradient of 10 to 80% ethyl

acetate/hexanes afforded (1) as a white foam; 0.403 g, 50% yield. ^{1}H NMR ($CDCl_3$): 8.88 (br s, 1H, NH), 8.81 (s, 1H, H8), 8.31 (s, 1H, H2), 8.11-7.53 (m, 5H, benzoyl), 6.18 (d, $J_{1',2'}$=4.8, 1H, H1'), 5.41 (t, $J_{3',2'}$=4.8, $J_{3',4'}$=4.8, 1H, H3'), 4.75 (t, $J_{2',1'}$=4.8, $J_{2',3'}$=4.8, 1H, H2'), 4.50-4.34 (m, 3H, H4', H5', H5''), 3.44 (s, 3H, OCH_3), 2.19 (s, 3H, OAc), 2.14 (s, 3H, OAc).

2'-O-Methyl-2-Amino-6-Chloropurine Riboside (5)

Sodium hydride (0.44g, 18.2 mmol) was added to the cooled (-20°C) solution of 2-amino-6-chloropurine riboside 4 (5g, 16.6 mmol) in dry dimethylformamide (100 mL) under stirring. After 1 hour the solution of CH_3I (1.24 mL, 19.9 mmol) in dry dichloromethane (10 mL) was added dropwise to the reaction mixture during 1 hour. Resulted yellow solution was stirred at -20°C for additional 2 hours until TLC (methylene chloride-methanol 9:1) showed complete disappearance of starting material. Reaction mixture was quenched with methanol (20 mL), warmed to the room temperature and evaporated to dryness *in vacuo*. The residue was dissolved in water (200 mL) and extracted with methylene chloride (3x200 mL). Organic layer was back extracted with water (100 mL). Combined aqueous phase was evaporated to dryness and the residue was purified by flash chromatography on silica using gradient of MeOH (7% to 10%) in methylene chloride to give 3.4g (65%) of the compound 5 . Flash chromatography purification can be substituted by multiple crystallization from acetonitrile, m.p. . Calcd. for $C_{10}H_{12}N_5O_4Cl$ (301.69): C 39.81; H 4.01; N 23.21; Cl 11.75 found C ; H ; N ; Cl . ^{1}H-NMR (DMSO-d_6): 3.32 (3H, s, 2'-OMe); 3.578 (1H, dd, 5'-H, $J_{4',5'}$ 4.0, $J_{5',5'}$ 12.0); 3.66 (1H, dd, 5'-H, $J_{4',5'}$ 4.0, $J_{5',5'}$ 12.0); 3.949 (1H, dd, 4'-H, $J_{3',4'}$ 3.6); 4.252 (1H, t, 2'-H, $J_{2',3'}$ 4.0); 4.312 (1H, m, 3'-H); 5.073 (1H, bs, 3'-OH, exchangeable); 5.236 (1H, bs, 5'-OH, exchang-eable); 5.908 (1H, d, 1'-H, $J_{1',2'}$ 6.0); 6.964 (2H, bs, 2-NH_2); 8.391 (1H, s, 8-H).

2'-O-Methyl Guanosine (9)

A mixture of 5 (2.05g, 6.5 mmol) , 1,4-diazabicyclo[2.2.2]octane (1 equiv.) and water (30 mL) was heated to 90°C for 45 minutes. Then the solution was cooled to ambient temperature, basified to pH 12 with 2M NaOH, and washed with methylene chloride (3x60 mL). The aqueous phase was acidified to pH 6 with 6M HCl and left at refrigerator overnight. Formed

precipitate was filtered off. Mother liquor was evaporated to dryness, the residue was dissolved in water and applied to the short column with RP-18 silica gel. Solid phase was washed with water and remaining product was eluted with 5% aq methanol. Appropriate fractions were combined, evaporated to dryness and recrystallized from to provide 1.25g (65%) of 2'-O-methyl guanosine 9. Analytical sample was recrystalized from water, m.p. The product was identical to authentic sample by HPLC, UV, ^{1}H-NMR-spectroscopy.

3',5'-di-O-Acetyl-2'-O-Methyl-6-Chloropurine Riboside (7)

To the solution of compound 5 (1.25g, 3.96 mmol), 4-dimethylaminopyridine (39 mg, 0.32 mmol) and triethylamine (0.37 mL, 2.64 mmol) in dry acetonitrile was added acetic anhydride (0.9 mL, 9.5 mmol) and the reaction mixture was left at room temperature for 40 minutes. Then it was quenched with MeOH (10 mL) and evaporated to dryness. The residue was dissolved in methylene chloride and washed with 1% aq acetic acid, saturated aq sodium bicarbonate, and brine. The organic layer was dried over sodium sulfate and evaporated to dryness yielding acetate 6. The residue was additionally dried in vacuo for 3 hours, dissolved in dry THF and degassed with dry argon. To the above boiling solution under positive pressure of argon isoamylnitrite (10 eq) was added dropwise. After 2 hours solvent was removed in vacuo and the residue was dissolved in methylene chloride, washed with saturated aq $NaHCO_3$ and brine. The residue after evaporation of organic phase was purified by flash chromatography on silica gel. Elution with hexanes-ethyl acetate (1:1) mixture provided 1.1g (72%) of compound 7 as yellow oil. Calcd. for $C_{15}H_{17}N_4O_6Cl$ (384.78): C 46.82; H 4.45; N 14.56; Cl 9.21; found C ; H ; N ; Cl ^{1}H-NMR ($CHCl_3$-d): δ 2.133 (3H, s, 3'-OAc or 5'-OAc); 2.184 (3H, s, 3'-OAc or 5'-OAc); 3.44 (3H, s, 2'-OCH_3); 4.425 (3H, m, 4'-H, 5'-CH_2); 4.686 (1H, t, 2'-H, $J_{2',3'}$ 5.04); 5.36 (1H, t, 3'-H, $J_{3',4'}$ 4.28); 6.132 (1H, d, 1'-H, $J_{1',2'}$ 4.88); 8.311 (1H, s, 8-H); 8.771(1H, s, 2-H).

2'-O-Methyl Adenosine (8)

Solution of compound 7 (0.45g, 1.17 mmol) in saturated methanolic ammonia (20 mL) was autoclaved at 125°C for 4 hours. The solvent was removed in vacuo and remaining residue was purified by flash

chromatography on silica gel. Elution with methylene chloride - methanol (9:1) mixture provided 0.25g (80%) of 2'-O-methyl adenosine **8** as white solid. The analytical sample was recrystallized from abs EtOH. m.p. The product was identical to authentic sample by HPLC, UV- , ^{1}H-NMR-spectroscopy.

2,6-Diamino-9-[3',5'-O-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl]purine (11):

To an oven baked 500 ml three neck round bottom flask equviped with mechanical stirrer, positive pressure argon, and rubber septum was added 2,6-Diamino-9-(β-D-ribofuranosyl)purine **(10)** (10.0 g, 35.4 mmol), anhydrous DMF (100 ml), and anhydrous pyridine (200 ml). The resulting light brown suspension was cooled to 0°C in an ice/water bath while stirring. TIPSCl (42.48 mmol, 13.6 ml) was added dropwise to the stirred 0°C reaction mixture via syringe over a 20 minute period. The reaction mixture was then warmed to rt resulting in a homogenious solution. TLC indicated complete reaction after 3 hours at rt, at which time the reaction was quenched by addition of ethanol (20 ml). The reaction mixture was then evaporated *in vacuo* and the resulting residue partitioned between ethyl acetate and sat. aqueous $NaHCO_3$ at which time (**11**) precipitated from the organic layer. The aqueous layer was then back extracted with ethyl acetate and the combined orgainics cooled to 0°C. The precipitate was filtered and washed with ethyl acetate to afford (**11**) as a beige solid; 16.5 g, 89% yield. ^{1}H NMR (dmso-d6): 7.77 (s, 1H, H8), 6.75 (s, exch, 2H, N^6-NH_2), 5.74 (s, exch, 2H, N^2-NH_2), 5.71 (s, 1H, H1'), 5.56 (d, $J_{OH,2'}$=5.0, 1H, 2'-OH), 4.43 (dd, $J_{3',2'}$=4.5, $J_{3',4'}$= 7.8, 1H, H3'), 4.29 (m, $J_{2',OH}$=5.0), 4.06-3.88 (m, 3H, H4', H5', H5''), 1.04 (m, 28H, TIPDS).

2,6-Diamino-9-[3',5'-O-tetraisopropyldisiloxane-1,3-diyl)-2'-O-methyl-β-D-ribofuranosyl]purine (12):

To an oven baked 500 ml three neck round bottom flask equiped with mechanical stirrer and positive pressure argon was added (**11**) (15.6 g, 29.7 mmol) followed by anhydrous DMF (300 ml) and methyl iodide (89.2 mmol, 5.55 ml). The reaction mixture was cooled to 0°C in an ice/water bath and 60% sodium hydride in oil (44.6 mmol, 1.78 g) added slowly. A temperature of 0°C was maintained for 35 minutes at which time the reaction was quenched with anhydrous ethanol and diluted into 2 volumes of 0°C dichloromethane.

The dilute reaction mixture was washed two times with sat. NH_4Cl, the aqueous layer back extracted with dichloromethane, and the combined organics dried over Na_2SO_4, filtered and evaporated to dryness *in vacuo*. Crystallization from ethanol/water 1:1 afforded 14.7 grams of (**12**), 92% yield. ^1H NMR (dmso-d6): 7.75 (s, 1H, H8), 6.76 (s, exch, 2H, N^6-NH_2), 5.78 (s, 1H, H1'), 5.73 (s, exch, 2H, N^2-NH_2), 4.58 (dd, $J_{3',2'}$=4.8, $J_{3',4'}$= 4.8, 1H, H3'), 4.12 (d, $J_{2',3'}$=4.8), 4.09-3.91 (m, 3H, H4', H5', H5''), 3.54 (s, 3H, OCH_3), 1.03 (m, 28H, TIPDS).

2,6-Diamino-N^2-isobutyryl-9-[3',5'-O-tetraisopropyldisiloxane-1,3-diyl)-2'-O-methyl-β-D-ribofuranosyl]purine (13):

A solution of (3) (0.5 g, 0.93 mmol) in anhydrous pyridine (20 ml) was cooled to -10°C in an ice/ethanol bath while stirring under argon. Isobutyryl chloride (1.02 mmol, 0.11 ml) was added dropwise to the stirred -10°C solution over a period of 5 minutes. The reaction mixture was stirred at -10°C for 2 hours followed by 1 hour at rt then quenched with ethanol (2 ml). After evaporating the reaction mixture to dryness *in vacuo*, the resulting residue was partitioned between dichloromethane and sat. aqueous $NaHCO_3$. The aqueous layer was back extracted with dichloromethane and the combined organics dried over Na_2SO_4. Filtration and evaporation of the filtrate *in vacuo* afforded a beige foam. Flash chromatography using a gradient of 2-4% ethanol in dichloromethane afforded (**13**) as a white foam; 0.55g, 97% yield. ^1H NMR (dmso-d6): 9.76 (s, exch, 1H, N^2-NH), 8.04 (s, 1H, H8), 7.20 (s, exch, 2H, N^6-NH_2), 5.88 (s, 1H, H1'), 4.71 (dd, $J_{3',2'}$=5.2, $J_{3',4'}$= 5.2, 1H, H3'), 4.26 (d, $J_{2',3'}$=5.2), 4.15-3.91 (m, 3H, H4', H5', H5''), 3.55 (s, 3H, OCH_3), 2.87 (m, 1H, iBu-CH), 1.06-0.96 (m, 34H, TIPDS, iBu-$(CH_3)_2$).

N^2-isobutylryl-2'-O-methylguanosine (14):

A solution of (**13**) (5.0 g, 9.28 mmol) in anhydrous pyridine (100 ml) was cooled to -10°C in an ice/ethanol bath while stirring under argon. Isobutyryl chloride (10.21 mmol, 1.07 ml) was added dropwise to the stirred -10°C solution over a period of 30 minutes. The reaction mixture was stirred at -10°C for 2 hours followed by 1 hour at rt then quenched with ethanol (20 ml). After evaporating the reaction mixture to dryness *in vacuo*, the resulting residue was partitioned between dichloromethane and sat. $NaHCO_3$. The aqueous

layer was back extracted with dichloromethane and the combined organics dried over Na_2SO_4. Filtration and evaporation of the filtrate *in vacuo* afforded a beige foam which was dissolved in glacial acetic acid (80 ml). To the stirred acetic acid solution was added water (40 ml) followed by $NaNO_2$ (74.24 mmol, 5.12 g). Another portion of $NaNO_2$ (74.24 mmol, 5.12 g) was added after 30 minutes and the reaction stirred at rt for 48 hours. The reaction mixture was diluted with one volume of n-butanol and evaporated *in vacuo* to 50% of the original volume. Co-evaporation with n-butanol (3X) was followed by partitioning the crude syrup between ethyl acetate and sat. aqueous $NaHCO_3$. After back extracting the aq. layer with ethyl acetate, the combined organics were evaporated to dryness *in vacuo* . The crude residue was then dissolved in anhydrous dichloromethane (50 ml) and treated with a solution of TEA•3HF (27.84 mmol, 4.54 ml) and TEA (8.17 ml), in dichloromethane (20 ml). The reaction mixture was evaporated to dryness *in vacuo* and subsequently dissolved in additional dichloromethane (20 ml). Evaporation followed by dilution was repeated 3 times, and the crude product purified by flash chromatography. A gradient of 2-10% ethanol in dichloromethane afforded (14) as light yellow foam; 3.02 g, 88% yield. ^{1}H NMR (dmso-d6): 12.08 (s, exch, 1H, NH), 11.63 (s, exch, 1H, NH), 8.29 (s, 1H, H8), 5.90 (d, $J_{1',2'}$=6.3, 1H, H1'), 5.23 (d, $J_{OH,3'}$=4.9, 1H, 3'-OH), 5.07 (t, $J_{OH,5'}$ = 5.3, $J_{OH,5''}$=5.3 , 1H, 5'-OH), 4.30 (m, $J_{3',2'}$=4.8, $J_{3',4'}$=3.3, 1H, H3'), 4.22 (t, $J_{2',1'}$=6.3, $J_{2',3'}$=4.8, 1H, H2'), 3.93 (m, $J_{4',3'}$=3.3, $J_{4',5'}$=3.9, $J_{4',5''}$=3.9, 1H, H4') 3.65-3.53 (m, 2H, H5', H5''), 3.33 (s, 3H, OCH_3), 2.78 (m, 1H, iBu-CH), 1.12 (d, 6H, iBu-$(CH_3)_2$).

N^2-isopropylphenoxyacetyl-2'-O-methylguanosine (15):

A solution of (12) (47.4 g, 88 mmol) in anhydrous pyridine (500 ml) was cooled to -10°C in an ice/ethanol bath while stirring under argon. Isopropylphenoxyacetyl chloride (96.8 mmol, 20.6 ml) was added dropwise to the stirred -10°C solution over a period of 5 minutes. The reaction mixture was stirred at -10°C for 2 hours followed by 1 hour at rt then quenched with ethanol (20 ml). After evaporating the reaction mixture to dryness *in vacuo*, the resulting residue was partitioned between dichloromethane and sat. aqueous $NaHCO_3$. The aqueous layer was back extracted with dichloromethane and the combined organics dried over Na_2SO_4. Filtration and evaporation of the filtrate *in vacuo* afforded a beige foam which was dissolved in glacial acetic

acid (1000 ml). To the stirred acetic acid solution was added water (400 ml) followed by $NaNO_2$ (742.4 mmol, 51.2 g). Another portion of $NaNO_2$ (742.4 mmol, 51.2 g) was added after 30 minutes and the reaction stirred at rt for 48 hours. The reaction mixture was diluted with one volume of n-butanol and evaporated *in vacuo* to 50% of the original volume. Co-evaporation with n-butanol (3X) was followed by partitioning the crude syrup between ethyl acetate and sat. aqueous $NaHCO_3$. After back extracting the aq. layer with ethyl acetate, the combined organics were evaporated to dryness *in vacuo*. The crude residue was then dissolved in anhydrous dichloromethane (500 ml) and treated with a solution of TEA•3HF (278.4 mmol, 45.4 ml) and TEA (81.7 ml), in dichloromethane (200 ml). The reaction mixture was evaporated to dryness *in vacuo* and subsequently dissolved in additional dichloromethane (200 ml). Evaporation followed by dilution was repeated 3 times, and the crude product purified by flash chromatography. A gradient of 2-10% ethanol in dichloromethane afforded (5) as light yellow foam; 29.2 g, 85% yield. ^1H NMR (dmso-d6): 11.65 (s, exch, 2H, NH, NH), 8.30 (s, 1H, H8), 7.18-6.88 (dd, 4H, phenoxy), 5.91 (d, $J_{1',2'}$=6.0, 1H, H1'), 5.24 (d, $J_{OH,3'}$=4.8, 1H, 3'-OH), 5.09 (t, $J_{OH,5'}$ = 5.6, $J_{OH,5''}$=5.2 , 1H, 5'-OH), 4.82 (s, 2H, CH_2), 4.31 (m, $J_{3',2'}$=4.8, $J_{3',4'}$=3.6, 1H, H3'), 4.23 (t, $J_{2',1'}$=6.0, $J_{2',3'}$=4.8, 1H, H2'), 3.93 (m, $J_{4',3'}$=3.6, $J_{4',5'}$=4.0, $J_{4',5''}$=3.9, 1H, H4') 3.65-3.53 (m, 2H, H5', H5''), 3.35 (s, 3H, OCH_3), 2.84 (m, 1H, iPr-CH), 1.17 (d, 6H, iPr-$(CH_3)_2$).

N1-Benzyl guanosine (16)

Guanosine hydrate (50 grams, 177mmol) was coevaporated twice from dimethylformamide (2 x 250ml) and dissolved in dry dmf (400 mls). N,N-dimethylformamide dibenzyl acetal was added (240 grams, 230 ml, 885 mmol) and the solution was heated with stirring to 80 °C for 18 hrs. The excess acetal was removed by steam distillation on a rotary evaporator. The product was recovered without chromatography by washing with dichloromethane / hexanes (1:1 v/v) to yield 84 g of the ortho-amide intermediate. The o ortho-amide was cleaved by treatment with aqueous sodium hydroxide (2N, 133 ml) at room temperature for four hours. The product was recrystallized from boiling water to yield 54 g (145 mmol, 82%) of pure (16). ^1H NMR (dmso-d_6): 7.97 (s, 1H, H8), 7.29 (m, 5H, Bz), 7.02 (bs, 2H, $2NH_2$), 5.70 (d, $J_{1',2'}$=5.6, 1H, H1'), 5.42 (bs, 1H, 2'OH), 5.23 (s, 1H, CH_2-Bz), 5.15 (bs, 1H, 3'OH), 5.00 (bs,

1H, 5'-OH), 4.41 (t, $J_{3',2'}$=5.6, $J_{3',4'}$=4.0, 1H, H3'), 4.08 (t, $J_{2',1'}$=6.3, $J_{2',3'}$=4.8, 1H, H2'), 3.85 (m, $J_{4',3'}$=3.5, $J_{4',5'}$=4.0, 1H, H4') 3.58-3.49 (m, 2H, H5', H5'').

N1-Benzyl-2'-O-Methyl Guanosine (17)

A 1 L pear shaped recovery flask with stir bar was charged with a mixture of **16** (50g, 134 mmol) , silver acetylacetonate (41 g 200mmol), TMSH (200 ml of 1N solution in methanol) and dimethylformamide (400 ml). The flask was heated to 70°C for two hours. The solution was cooled to ambient temperature, neutralized to pH 7 with 1M HCl, and dried to a solid tar. The tar was dissolved in water (500ml) and filtered through a sintered glass funnel to remove silver salts. The product was evaporated to dryness to remove water and residual solvents and 10 grams (26mmols) was redissolved in 50 ml of water prior to purification on a Waters Delta-Pak ODS 50mm x 300mm HPLC column. The N1-Bz-2'-OMe guanosine isomer eluted first and was recovered using a rotary evaporator. The product (7 g, 18mm, 70%) was identical to an authentic sample by HPLC, UV- , ^{1}H-NMR-spectroscopy. ^{1}H NMR (dmso-d_6): 8.02 (s, 1H, H8), 7.26 (m, 5H, ph), 7.02 (bs, 2H, 2NH2), 5.82 (d, $J_{1',2'}$=6.4, 1H, H1'), 5.23 (s, 2H, CH2-Bz), 5.18 (d, $J_{OH,3'}$=4.8, 1H, 3'-OH), 5.04 (t, $J_{OH,5'}$ = 5.2, $J_{OH,5''}$=5.6 , 1H, 5'-OH), 4.28 (m, $J_{3',2'}$=4.8, $J_{3',4'}$=3.2, 1H, H3'), 4.20 (t, $J_{2',1'}$=6.4, $J_{2',3'}$=4.8, 1H, H2'), 3.90 (m, $J_{4',3'}$=3.6, $J_{4',5'}$=4.0, 1H, H4'), 3.65-3.53 (m, 2H, H5', H5'' $J_{OH,5''}$=5.2, $J_{5',5''}$=11.6), 3.33 (s, 3H, OCH_3).

2'-O-Methyl Guanosine (9)

Sodium spheres (3.5 grams) in mineral oil were washed with hexanes and weighed into dry THF. A glass bottle with polyethylene closure was charged with naphthalene (21.2 g, scintillation grade), dry THF (210 ml) and a glass sealed stir bar. The sodium was added and the mixture was stirred vigorously for one hour. The solution turned dark green after ten minutes and all sodium was presumed to be consumed after one hour to yield 150 mm of 0.6M sodium naphthalene solution. This solution was used without further characterization. A 250 ml flask was charged with N_1Bzl-2'-O-methyl guanosine (2 g, 5.0 mm) and a glass sealed stir bar. Sodium naphthalene solution was added (50mmol, 90 ml) and the solution was stirred overnight. TLC (10%MeOH in DCM) showed complete deblock of the benzyl group. The reaction was quenched with 10 mls of methanol and all solvents were

removed with a rotary evaporator. Water (100 ml) was added and the solution was neutralized with HCl (1N, pH 7, 50ml). Naphthalene was removed with extraction by toluene (3 x 100 mls) and the solution was pumped over an ODS Delta-Pak column to recover 2'O-Me guanosine nucleoside. The product (1.3 g, 4.5mmol, 90%) was identical to an authentic sample by HPLC, UV-Vis & ^{1}H-NMR-spectroscopy.

Trimethylsulfonium Hydroxide (TMSH) Solution in Methanol:

A 0.2 M solution of trimethyl sulfonium iodide (TMSI, 102 grams, 0.5 mol), in 2.5 L of methanol and water (9:1, v/v) was prepared by heating to 40 C and mixing continuously for 30 min. The clear, colorless solution was allowed to cool to r.t. A glass chromatography column (Ace #50 thread; 75mm x 300mm) containing Duolite 147 anion exchange resin (900 g) in the hydroxide form was previously packed and used for the conversion of TMSI to TMSH. The solution was pumped over the column using a gear pump at 100 ml per minute. The resin was washed with an additional 1 liter of 90% methanol and the total 3.5 liters was reduced in volume to 500 ml using a rotary evaporator with the bath set at 20 °C. The solution was checked for the presence of iodide ion using acidified silver nitrate solution and found to be negative. The solution was not characterized further and was stored in a teflon bottle with a gas vent at 5 °C.

2'-O-Methyl Adenosine (8)

A solution of adenosine (50 g, 187mmol) in dimethylformamide (400 ml) was prepared by heating to 50 °C with continuous mixing for 10 minutes in a 1 L pear shaped recovery flask containing a stir bar. Silver acetylacetonate (58 g, 280 mmol) and TMSH (280 ml of a 1 M) solution was added and the mixture was stirred immediately. The reaction mixture was heated to 75 °C with stirring for 45 minutes. A sample of the mixture showed no starting adenosine and two spots of which the predominant one comigrates with an authentic sample of 2'-OMe adenosine. An HPLC assay showed an nine to one ratio for 2' to 3'-OMe adenosine The solvent was removed by rotary evaporation and 500 ml of water and 250 ml of 1N HCl was added to neutralize the hydroxide (pH 7 by Hydrion strips) prior to filtration on a sintered glass funnel. The water and solvents were removed by rotary evaporation leaving a brown tar. This

material was purified on a ODS 25mm x 300mm Delta-Pak HPLC column using water as the eluant. Overall recovery of 42 grams for a yield of 75% from adenosine. The product **12** was identical to authentic sample by HPLC, UV- , ^{1}H-NMR-spectroscopy.

These examples are meant to be non-limiting and those skilled in the art will recognize that similar strategies, as described in the present invention, can be readily adapted to synthesize other methoxy nucleosides and nucleoside analogs and are within the scope of this invention.

Methods

The following are examples of preferred embodiments of the present invention. Those in the art will recognize that these are not limiting examples but rather are provided to guide those in the art to the full breadth of meaning of the present invention. Routine procedures can be used to utilize other coupling regions not exemplified below.

Ribozymes were synthesized in two parts and tested without ligation for catalytic activity. Referring to Fig. 20, the cleavage activity of the half ribozymes containing between 5 and 8 base pairs stem IIs at 40 nM under single turnover conditions was comparable to that of the full length oligomer as shown in Figs. 21 and 22. The same half ribozymes were synthesized with suitable modifications at the nascent stem II loop to allow for crosslinking. The halves were purified and chemically ligated, using a variety of crosslinking methods. The resulting full length ribozymes (see Fig. 19) exhibited similar cleavage activity as the linearly synthesized full length oligomer as shown in Fig. 22.

Synthesis, Deprotection and Purification of RNA

Synthesis of RNA

The general procedures for RNA synthesis have been described previously (Usman et al., (1987) *J. Am. Chem. Soc.*, **109**, 7845-7854 and Scaringe et al., *supra*; Wincott et al., 1995 *Nucleic Acids Res.* in press). Small scale syntheses were conducted on a 394 (ABI) synthesizer

using a modified 2.5 µmol scale protocol with a 5 min coupling step for alkylsilyl protected nucleotides and 2.5 min coupling step for 2'-*O*-methylated nucleotides. Table II outlines the amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163 µL of 0.1 M = 16.3 µmol) of phosphoramidite and a 24-fold excess of *S*-ethyl tetrazole (238 µL of 0.25 M = 59.5 µmol) relative to polymer-bound 5'-hydroxyl was used in each coupling cycle. Average coupling yields on the 394, determined by colorimetric quantitation of the trityl fractions, was 97.5-99%. Other oligonucleotide synthesis reagents for the 394: Detritylation solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% *N*-Methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I2, 49 mM pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile was used directly from the reagent bottle. *S*-Ethyl tetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from American International Chemical, Inc.

RNA Deprotection of Exocyclic Amino Protecting Groups Using Methylamine (MA)

The polymer-bound oligoribonucleotide, trityl-off, was transferred from the synthesis column to a 4 mL glass screw top vial and suspended in a solution of methylamine (MA) at 65 °C for 10 min. After cooling to -20 °C, the supernatant was removed from the polymer support. The support was washed three times with 1.0 mL of EtOH:MeCN:H_2O/3:1:1, vortexed and the supernatant was then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, were dried to a white powder.

Trityl-off RNA Deprotection of 2'-Hydroxyl Alkylsilyl Protecting Groups Using Anhydrous TEA•3HF

The base-deprotected oligoribonucleotide was resuspended in anhydrous TEA•HF/NMP solution (250 µL of a solution of 1.5 mL *N*-methylpyrrolidinone, 750 µL TEA and 1.0 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C for 1.5 h. The resulting, fully deprotected, oligomer was quenched with 50 mM TEAB (9 mL) prior to anion exchange desalting.

For anion exchange desalting of the deprotected oligomer, the TEAB solution was loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that was prewashed with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA was eluted with 2 M TEAB (10 mL) and dried down to a white powder (Scaringe et al., 1990 *Nucleic Acids Res.*, 18, 5433).

RNA Purification

For a small scale (2.5 µmol) synthesis, the crude material was diluted to 5 mL with RNase free water. The sample was injected onto either a Pharmacia Mono Q® 16/10 mm or Dionex NucleoPac® PA-100 22 x 250 mm column with 100% buffer A (10 mM $NaClO_4$). A gradient from 180-210 mM $NaClO_4$ at a rate of 8 mL/min for a Pharmacia Mono Q anion-exchange column or 100-150 mM $NaClO_4$ at a rate of 15 mL/min for a Dionex NucleoPac® anion-exchange column was used to elute the RNA. Fractions were analyzed by HPLC and those containing full length product ~80% by peak area were pooled for desalting. The pooled fractions were applied to a SepPak cartridge (C_{18}) that was prewashed successively with CH_3CN (10 mL), CH_3CN/MeOH/H_2O:1/1/1 (10 mL) and RNase free H_2O (20 mL). Following sample application, the cartridge was washed with RNase free H_2O (10 mL) to remove the salt. Product was then eluted from the column with CH_3CN/MeOH/H_2O:1/1/1 (10 mL) and dried.

Biochemical Activity

Ribozyme Activity Assay

Ribozymes and 5'-^{32}P-end-labeled substrate were heated separately in reaction buffer (50 mM Tris-Cl, pH 7.5; 10 mM $MgCl_2$) to 95 °C for 2 min, quenched on ice, and equilibrated to 37 °C prior to starting the reactions. Reactions were carried out in enzyme excess, and were started by mixing ~1 nM substrate and 40 nM ribozyme to a final volume of 50 µL. Aliquots of 5 µL were removed at 1, 5, 15, 30, 60 and 120 min, quenched in formamide loading buffer, and loaded onto 15% polyacrylamide/8 M urea gels. The fraction of substrate and product present at each time point was determined by quantitation of scanned images from a Molecular Dynamics

PhosphorImager®. Ribozyme cleavage rates were calculated from plots of the fraction of substrate remaining *vs* time using a double exponential curve fit (Kaleidagraph, Synergy Software).

Example 10

Referring to Fig. 18 the 5' half of a hammerhead ribozyme was provided with a ribose group. This was oxidatively cleaved with $NaIO_4$ and reacted with the 3' half of the ribozyme having an amino group under reducing conditions. The resulting ribozyme consisted of the two half ribozyme linked by a morpholino group.

One equivalent of (200 micrograms) of RPI3631 (5' half hammerhead with a 3'OH) and 5 equivalents (1000 micrograms) of RPI3631 (3' half with 5' C5-NH_2) all with RPI 3635 (3' half hammerhead ribozyme with 5' C5-NH_2) were used in this reaction. The limiting oligonucleotide was oxidized first with 3.6 equivalents of sodium periodate for sixty minutes on ice in DEPC water quenched with 7.2 equivalents of ethylene glycol for 30 minutes on ice and the 5 equivalents of the amino oligo added. 0.5 Molar tricine buffer, pH 9, was added to provide 25 millimolar final tricine concentration and left for 30 minutes on ice. 50 equivalents of sodium cyanoborohydride was then added and the pH reduced to 6.5 with acetic acid and reaction left for 60 minutes on ice. The resulting full length ribozyme was then purified for further analysis.

Example 11: Amide Bond

Referring again to Fig. 18 and 19, a 5' half of ribozyme was provided with a carboxyl group at its 2' position and was coupled with an amine containing 3' half ribozyme. The provision of a coupling reagent resulted in a full-length ribozyme having an amide bond.

Example 12: Disulfide Bond

Referring to Fig. 18 and 19, 250 micrograms of RPI3881 and 250 micrograms of RPI3636 half ribozyme were separately deprotected with dithiothreitol overnight at 37°C. They were mixed together at 1:1 mole ratio in a 100 mM sodium phosphate buffer at pH 8 and 4M copper sulfate and 0.8 mM 1,10-phenanthroline (final concentrations) was added for two hours at

room temperature (20-25°C) and the resulting mixture gel purified. The overall purification yield of full length ribozyme was 30%.

Example 13: Synthesis And Activity Of Morpholino-Linked Ribozymes

Analogs of RPI.2972 (Table IV) were synthesized as described above. RPI.2972 is a chemically stabilized ribozyme targeted against site 575 of *c-myb* mRNA that inhibits smooth muscle cell proliferation with an IC_{50} of approximately 75 nM (Stinchcomb *et al., International PCT Publication No.* WO 95/31541. Referring to Figure 23, the half-ribozymes, 1 and 2, used in this study contained a modified 5 base-paired stem II and the appropriate reactive groups at the termini.

Half-ribozymes 1 and 2 were synthesized and purified according standard methods. The 3'-uridilyl-5'-half-ribozyme, 1, (150 μM) was dissolved in sodium N_2-acetamido-2-imino-diacetate (ADA) buffer (100 mM, pH 6.0) and subjected to oxidative cleavage with 2 molar equivalents of a 100 mM aqueous solution of sodium periodate (Figure 24A). After 2 h, the acyclic 2',3'-dialdehyde derivative 4 was formed quantitatively, as confirmed by HPLC monitoring (Figure 24B). ES-MS analysis, performed on purified 1 (calc. 6490.2, found 6489.1) and 4 (calc. 6488.2, found 6489.2), did not allow direct identification since 1 and 4 differed only by two atomic mass units (amu). However, the presence of the 3'-phosphoryl-5'-half-ribozyme, 5, (calc. 6263.1, found 6262.6) in the ES-mass spectrum of 4 provided supportive evidence. Indeed, 5 confirmed the dialdehydic structure of 4 since it resulted from the E_2-elimination of 2',3'-dideoxy-2',3'-diformyl-5'-deoxy-5'-methylene-uridine occuring during the ammonium acetate precipitation of 4 (Figure 24A). As expected, 5 (retention time = 5.3 min) was never observed in non-desalted samples of 4 (retention time = 5.5 min) (Figure 24C) thus corroborating that the ammonium acetate pH 8 precipitation was responsible for the base-catalyzed β-elimination.

The crude, oxidized mixture containing 4 and an excess of $NaIO_4$ was then directly mixed with the 5'-aminohexyl-3'-half-ribozyme 2 under reductive amination conditions (Figure 25). The unreacted sodium periodate was not quenched with a *cis*-diol source as the resulting aldehydes might have competed with 4 in the reductive alkylation reaction of 2. Moreover, since the

3'-end of **2** contained an inverted abasic residue, no *cis*-diol functionalities susceptible to undesired oxidative cleavage were present. Typically, **2** (600 μM) was added to the crude **4** in 100 mM ADA buffer pH 6.0. The transient Schiff base adduct could not be formed unless a 5 molar excess of aqueous $NaBH_3CN$ (500 mM) was introduced (Figure 25) leading to the concomitant formation of the cross-linked products **6** and **7** in a 3 to 1 ratio. Reducing the molar excess of **2** from 4 eq. to nearly stoichiometric (1.5 eq.) did not change the course of the reaction. After purification, **6** was identified as the morpholino-linked ribozyme on the basis of ES-MS analysis (calc. 11723.7, found 11724.8). Interestingly, the ES-MS of compound **7** exhibited a mass signal higher than **6** by 38.8 amu (Figure 26). This suggested that the higher mass product **7** was a cyanoborane adduct of the tertiary nitrogen atom of the morpholino moiety (calc. 11762.7, found 11763.6). To confirm the identity of compound **7**, applicant prepared ^{13}C-labeled $NaBH_3{}^{13}CN$ from $Na^{13}CN$ according to the procedure of Hui (*Inorg. Chem.* **1980**, *19*, 3185-3186), and repeated the reductive amination of **4** on a 2 μmol scale. As expected, **6** and **7** were produced, ^{13}C-NMR performed on the two products clearly showed a singlet at 126.8 ppm for **7**, confirming the presence of a cyanoborane adduct whereas this signal could not be observed in the ^{13}C spectrum of **6**.

Once the morpholino-linked ribozymes were synthesized and characterized it was critical to ascertain the effect of this chemical cross-link on the rate of catalytic cleavage. Ribozymes **6** and **7** as well as the control RPI.2972 were assayed for their cleavage rate on short substrate **3** (Figure 23) as described above. The cleavage activity of the morpholino-linked ribozyme **6** was very similar to that of the control (Table IV), confirming that one can extensively modify the loop II/stem II region without hampering catalytic efficiency. Interestingly, **7** cleaved substrate **3** six times faster than the control, RPI.2972.

This general segmented assembly can be readily used to synthesize and assemble larger ribozyme motifs such as hairpin, Hepatitis Delta Virus or VS ribozymes.

Nucleotides and Nucleosides

Applicant has found that chemical modifications of this invention are particulary useful for enzymatic nucleic acid molecule stabilization. Thus, below is provided examples of one such molecule, a hammerhead ribozyme. Those in the art will recognize that equivalent procedures can be used to make other enzymatic nucleic acid molecules having a 5'- and/or 3'-cap structure. Specifically, Figures 1 and 27 show base numbering of a hammerhead motif in which the numbering of various nucleotides in a hammerhead ribozyme is provided. This is not to be taken as an indication that the Figure is prior art to the pending claims, or that the art discussed is prior art to those claims.

The following are non-limiting examples showing the synthesis and activity of enzymatic nucleic acids containing 5'- and/or 3'-cap modifications and the synthesis of monomer phosphoramidites.

Example 14: Synthesis of enzymatic nucleic acids containing 5'- and/or 3'-cap structures

The method of synthesis follows the procedure for normal RNA synthesis as described in Usman,N.; Ogilvie,K.K.; Jiang,M.-Y.; Cedergren,R.J. *J. Am. Chem. Soc.* 1987, *109*, 7845-7854; Scaringe,S.A.; Franklyn,C.; Usman,N. *Nucleic Acids Res.* 1990, *18*, 5433-5441; and Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677 (all of these references are incorporated by reference herein in their entirety) and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. Phosphoramidites of the 5'-cap and/or 3'-cap structures selected from those described and illustrated in Figures 28-29 and 32-38 may be incorporated not only into hammerhead ribozymes, but also into hairpin, hepatitis delta virus, VS RNA, RNase P ribozyme, Group I or Group II intron catalytic nucleic acids. They are, therefore, of general use in any enzymatic nucleic acid structure.

Example 15: Incorporation of 5'-Amino- and 5'-Mercapto-5'-Deoxy-2'-*O*-Methyl Nucleosides Into Hammerhead Ribozymes

Non-chiral phosphoramidate and phosphorothioate linkages (Figure 29) for incorporation at the 5'-end of a hammerhead ribozyme are described *infra*. These linkages are electronically and sterically similar to their natural congener and introduction of a single 3'-O-P(O)(O^-)-NH-5' or 3'-O-P(O)(O^-)-S-5' link at the 5'-end of the ribozyme has little effect on its hybridization to a substrate and/or ribozyme cleavage activity. Letsinger and Mungall, *J. Org. Chem.* 1970, *35*, 3800-3803, reported the synthesis of a thymidine dimer and trimer possessing internucleotide phosphoramidate bonds 3'-O-P(O)(O^-)-NH-5' which were stable in neutral and alkaline conditions and showed increased stability against exonucleases. The terminal 5'-amino group of a thymidine dimer was found to efficiently inhibit the action of spleen phosphodiesterase. It is also reported that introduction of a phosphoramidate 3'-NH-P(O)(O^-)-O-5' leads to enhancement in stability of the heteroduplex (Gryaznov and Letsinger, *Nucleic Acids Res.* 1992, *20*, 3403-3409). While studies of 3'-*S*-modified oligodeoxynucleotides demonstrated complete resistance to cleavage by *Eco*RV, there are no related studies on 5'-*S*-modified oligonucleotides (Vyle *et al.*, *Biochemistry* 1992, *31*, 3012-3018). Although there is interest in the synthesis, chemical and biological properties of oligonucleotides with bridging 5'-*N* (Letsinger *et al.*, *supra*; Mag and Engels, *Tetrahedron* 1994, *50*, 10225-10234; Gryaznov and Sokolova, *Tetrahedron Lett.* 1990, *31*, 3205-3208; Letsinger *et al.*, *Nucleic Acids Res.* 1976, *3*, 1053-1063; Mag, and Engels, *Nucleosides & Nucleotides* 1988, *7*, 725-728) and 5'-*S* (Sund and Chattopadhyaya, *Tetrahedron* 1989, *45*, 7523-7544; Chladek amnd Nagyvary, *Amer. Chem. Soc.* 1972, *94*, 2079-2085; Cook, *J. Amer. Chem. Soc.* 1970, *92*, 190-195; Liu and Reese, *Tetrahedron Lett.* 1995, *36*, 3413-3416) substitutions as well as 3'-*N* (Mag *et al.*, *Tetrahedron Lett.* 1992, *33*, 7319-7322; Zielinski and Orgel, *Nucleic Acids Res.* 1987, *15*, 1699-1715) and 3'-*S* (Cosstick and Vyle, *Nucleic Acids Res.* 1990, *18*, 829-835; Li *et al.*, *Tetrahedron* 1992, *48*, 2729-2738; and *J. Chem. Soc. Perkin I* 1994, 2123-2129; Liu and Reese, *Tetrahedron Lett.* 1996, *37*, 925-928) modified oligonucleotides, there are few reports (Bannwarth, *Helv. Chim. Acta* 1988, *71*, 1517-1427; Mag and Engels, *Nucleic Acids Res.* 1989, *17*, 5973-5988; Mag *et al.*, *Nucleic Acids Res.* 1991, *19*, 1437-1441; Chen *et al.*,

Nucleic Acids Res. 1995, *23*, 2661-2668; Cosstick and Vyle *Tetrahedron Lett.* 1989, *30*, 4693-4696) of the step-by-step elongation on solid support using 5'- or 3'-*N*(*S*)-modified nucleotide monomers.

Because of the different chemical nature of N-R and S-R bonds compared to O-R bonds there is a requirement for introduction of special protecting groups for amino and thiol functions and special conditions for their cleavage, considerably different from those routinely used in a solid phase nucleic acid synthesis, but still compatible with solid phase phosphoramidite chemistry. Also, optimization of the synthetic cycle for the introduction of the modified monomers is usually necessary.

Based on previous investigations in the 2'-deoxy series (Mag *et al.*, 1989 and 1991 *supra)* we have chosen 4-methoxytrityl (MMTr) group for the protection of the 5'-amino function while the trityl (Tr) group was used for the protection of the 5'-mercapto functionality in modified monomers.

The synthesis of 5'-amino-5'-deoxy-2'-*O*-methyl-uridine, guanosine and adenosine 3'-phosphoramidites **5**, **11** and **20** (Figure 29B and 29C), as well as 5'-mercapto-5'-deoxy-2'-*O*-methyl-uridine and cytidine 3'-phosphoramidite **23** and **28** (Figure 29D) and their incorporation into ribozymes are described *infra*. Extensive modification of hammerhead ribozyme with 2'-*O*-Me-nucleosides resulted in a catalytic motif with almost wild type cleavage activity and considerably improved nuclease stability has recently been described (Beigelman *et al.*, *J. Biol. Chem.* 1995, *270*, 25702-25708). Another reason for using 2'-*O*-methyl modified nucleotides is to prevent degradation of oligonucleotides by attack of the free neighboring 2'-hydroxyl on the phosphorus during deprotection, a well documented event in the case of 5'-*S*-modified ribonucleoside dimers.

MATERIALS AND METHODS

General methods

2'-*O*-Methyluridine, N^2-isobutyryl-2'-*O*-methylguanosine and 5'-*O*-(4,4'-dimethoxytrityl)-N^6-benzoyl-2'-*O*-methyladenosine were obtained from ChemGenes Corporation (Waltham, MA). All NMR spectra were recorded on

a Varian Gemini 400 spectrometer operating at 400.075 MHz for proton and 161.947 MHz for phosphorus. Chemical shifts in ppm refer to TMS and H_3PO_4, respectively. The solvent was $CDCl_3$ if not stated otherwise. The standard work up consisted of partitioning of the residue after removal of solvents between 5% aqueous $NaHCO_3$ and CH_2Cl_2 followed by washing of the organic layer with brine, drying over Na_2SO_4 and removal of solvents *in vacuo*. Analytical thin-layer chromatography (TLC) was performed with Merck Art. 5554 Kieselgel 60 F_{254} plates and column chromatography using Merck 0.040-0.063 mm Silica gel 60. Melting temperatures were determined on the Electrothermal Model IA 9200 apparatus and are uncorrected.

The general procedures for RNA synthesis and deprotection have been described previously (Wincott *et al., supra,* incorporated by reference herein in its entirety) Syntheses were conducted on a 394 (ABI) synthesizer using a modified 2.5 µmol scale protocol with a 5 min coupling step for 2'-*O*-TBDMSi protected nucleotides and 2.5 min coupling step for 2'-*O*-methyl nucleotides. A 6.5-fold excess of a 0.1 M solution phosphoramidite and a 24-fold excess of *S*-ethyl tetrazole relative to polymer-bound 5'-hydroxyl was used in each coupling cycle.

All analytical HPLC analyses were performed on a Hewlett Packard 1090 HPLC with a Dionex NucleoPac® PA-100 column, 4 x 250 mm, at 50 °C, as reported (Wincott *et al., supra*).

CGE analyses were performed on a Hewlett Packard ^{3D}CE with a J & W µPAGE ™ -5 (5% T, 5% C) polyacrylamide gel-filled column, 75µm I.D. x 75 cm, 50 cm effective length, 100 mM Tris-Borate, 7 M Urea, pH = 8.3, and J & W µPAGE ™ Buffer (100 mM Tris-Borate, 7 M Urea, pH = 8.3). Samples were electrokinetically injected using -13 kV for 3-10 sec, run at -13 kV and detected at 260 nm.

MALDI-TOF mass spectra were determined on a PerSeptive Biosystems Voyager spectrometer.

Synthesis of monomer building blocks

Referring to Figure 29B, *5'-Azido-5'-deoxy-2'-O-methyluridine (2)* was synthesized from 2'-*O*-methyluridine (1) in 79% yield (white foam) according to the procedure of Yamamoto *et al., J. Chem. Soc. Perkin I* 1978, 306-310 (incorporated by reference herin in its entirety), for the preparation of 5'-azido-5'-deoxythymidine, ^{1}H NMR δ 9.16 (br s, 1H, NH), 7.70 (d, $J_{6,5}$=8.2, 1H, H6), 5.97 (d, $J_{1',2'}$=2.2, 1H, H1'), 5.87 (d, $J_{5,6}$=8.2, 1H, H5), 4.21 (m, 1H,H3'), 4.08 (m, 1H, H2'), 3.92 (dd, $J_{5',4'}$=2.2, $J_{5',5''}$=13.4, 1H, H5'), 3.87 (dd, $J_{4',5'}$=2.2, $J_{4',3'}$=5.6, 1H, H4'), 3.82 (dd, $J_{5'',4'}$=3.3, $J_{5'',5'}$=13.4, 1H, H5''), 3.67 (s, 3H, OMe).

5'-Amino-5'-deoxy-2'-O-methyluridine (3) (Figure 29B) was synthesized from **2** according to a modification of the procedure of Mag and Engels, *Nucleic Acids Res.* 1989, *17*, 5973-5988 (incorporated by reference herin in its entirety), for the preparation of 5'-amino-5'-deoxythymidine: **2** (680 mg, 2.27 mmol) was dissolved in dry pyridine (5 mL) and triphenylphosphine (Ph_3P) (890 mg, 3.39 mmol) was added. The mixture was stirred for 2 h at rt at which time all the starting material had reacted. Concentrated NH_4OH (2 mL) was then added and the mixture stirred at rt for 2 h. Solvents were removed at reduced pressure, water was added (20 mL) and precipitate removed by filtration. The filtrate was extracted with benzene and ether and then evaporated to dryness. The residue was dissolved in isopropanol from which the amorphous solid precipitated on cooling (480 mg, 82%), ^{1}H NMR (dmso-d_6) δ 8.01 (d, $J_{6,5}$=8.1, 1H, H6), 5.90 (d, $J_{1',2'}$=5.2, 1H, H1'), 5.71 (d, $J_{5,6}$=8.1, 1H, H5), 4.16 (app t, $J_{3',4'}$=5.0, 1H, H3'), 3.91 (app t, $J_{2',1'}$=5.2, 1H, H2'), 3.84 (q, $J_{4',3'}$=5.0, 1H, H4'), 3.43 (s, 3H, OMe), 2.88 (dd, $J_{5',4'}$=4.5, $J_{5',5''}$=13.7, 1H, H5'), 2.83 (dd, $J_{5'',4'}$=5.0, $J_{5'',5'}$=13.7, 1H, H5'').

5'-N-(4-Methoxytrityl)amino-5'-deoxy-2'-O-methyluridine (4) (Figure 29B) was synthesized from **3** using 4-methoxytrityl chloride/DMAP/Et_3N/Pyr in 63% yield according to the procedure of Mag and Engels, *Nucleic Acids Res.* 1989, *17*, 5973-5988, and is incorporated by reference herin in its entirety. ^{1}H NMR δ 8.25 (br s, 1H, NH), 7.54-6.88 (m, 15H, aromatic, H6), 5.96 (s, 1H, H1'), 5.70 (d, $J_{5,6}$=7.9, 1H, H5), 4.13 (m, 1H, H3'), 4.01 (m, 1H, H2'), 3.86 (s, 3H, TrOMe), 3.77 (m, 1H, H4'), 3.69 (s, 3H, OMe), 2.82 (dd, $J_{5',4'}$=2.9, $J_{5',5''}$=12.9, 1H, H5'), 2.66 (d, $J_{NH,5'}$=8.8, 1H, 5'NH), 2.42 (dd, $J_{5'',4'}$=6.8, $J_{5'',5'}$=12.9, 1H, H5'').

5'-N-(4-Methoxytrityl)amino-5'-deoxy-2'-O-methyluridine-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (5). (see Figure 29B) To the solution of **4** (520 mg, 0.98 mmol) and *N,N*-diisopropylethylamine (DIPEA) (0.34 mL, 1.95 mmol) in CH_2Cl_2 (10 mL) under argon was added 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.30 mL, 1.34 mmol) was added dropwise, stirring was continued for 3 h at rt. The reaction mixture was then cooled to 0 °C, dry MeOH (3 mL) was added and stirring continued for 5 min. The mixture was evaporated to dryness *in vacuo* (40 °C bath temp) and the residue chromatographed on a silica gel column using 20-70% gradient EtOAc in hexane (1% Et_3N) to afford **5** as a colorless foam (0.60 g, 83%), ^{31}P NMR δ 148.97 (s) and 148.67 (s).

5'-O-p-Toluenesulfonyl-N²-isobutyryl-2'-O-methylguanosine (7). (see Figure 29B) *N²*-Isobutyryl-2'-*O*-methylguanosine (**6**) (Inoue *et al., Nucleic Acids Res.* 1987, *15*, 6131-6148, and is incorporated by reference herin in its entirety) (1.6 g, 4.36 mmol) was dissolved in dry pyridine (25 mL) and the solution was cooled to 0 °C while protected from moisture. p-Toluenesulfonyl chloride (1.0 g, 5.23 mmol) was added and the reaction mixture was left at 0-3 °C for 48 h. MeOH (10 mL) was added and the mixture evaporated to a syrup. After standard work up and column chromatography using 1-2% MeOH in CH_2Cl_2, **7** was obtained as a colorless foam, 1.06 g (47%), ^1H NMR δ 12.25 (br s, 1H, NH), 9.55 (br s, 1H, NH), 7.83 (d, $J_{H,H}$=8.3, 2H, Ts), 7.78 (s, 1H, H8), 7.42 (d, $J_{H,H}$=8.3, 2H, Ts), 5.83 (d, $J_{1',2'}$=6.2, 1H, H1'), 4.82 (app t, $J_{2',3'}$=5.7, 1H, H2'), 4.64 (m, 1H, H3'), 4.37 (dd, $J_{5',4'}$=2.2, $J_{5',5''}$=10.3, 1H, H5'), 5.23 (dd, $J_{4',5''}$=2.9, $J_{4',3'}$=5.2, 1H, H4'), 4.29 (dd, $J_{5'',4'}$=2.9, $J_{5'',5'}$=10.3, 1H, H5''), 3.47 (s, 3H, OMe), 2.76 (m, 1H, *CH*$(CH_3)_2$), 2.51 (s, 3H, Ts-Me), 1.29 (m, 6H, 2 x Me).

The 3',5'-Di-*O*-p-toluenesulfonyl derivative was also isolated (0.45 g, 15%) from the reaction mixture along with 20% of the unreacted starting material.

As shown in Figure 29B *5'-Azido-5'-deoxy-N²-isobutyryl-2'-O-methylguanosine (8).* **7** (780 mg, 1.5 mmol) was dissolved in dry DMSO (7 mL) and LiN_3 (370 mg, 7.56 mmol) was added under argon. The mixture was heated at 50 °C for 16 h and then evaporated to a syrup (oil pump, 50 °C). The

residue was partitioned between water (30 mL) and EtOAc (30 mL). The aqueous layer was extracted with EtOAc (4 x 20 mL), organic layers combined, dried (Na_2SO_4) and evaporated to dryness. Flash column silica gel chromatography using 2-25% MeOH in CH_2Cl_2 afforded **8**, 430 mg (78%), mp 107-109 °C (H_2O), ^1H NMR (dmso-d_6) δ 12.17 (br s, 1H, NH), 11.68 (br s, 1H, NH), 8.36 (s, 1H, H8), 6.01 (d, $J_{1',2'}$=6.1, 1H, H1'), 5.52 (d, $J_{OH,3'}$=5.1, 1H, 3'OH), 4.47 (app t, $J_{2',3'}$=5.5, 1H, H2'), 4.37 (m, 1H, H3'), 4.12 (m, 1H, H4'), 3.75 (dd, $J_{5',4'}$=6.8, $J_{5',5''}$=13.2, 1H, H5'), 3.65 (dd, $J_{5'',4'}$=4.2, $J_{5'',5'}$=13.2, 1H, H5"), 3.43 (s, 3H, *O*Me), 2.86 (m, 1H, *CH*$(CH_3)_2$), 1.22 (s, 3H, Me), 1.20 (s, 3H, Me).

5'-Amino-5'-deoxy-N^2-isobutyryl-2'-O-methylguanosine (9) (Figure 29B) To the solution of **8** (350 mg, 0.95 mmol) in 96% EtOH (30 mL) 10% Pd/C catalyst (60 mg) was added. The mixture was hydrogenated under 35 psi of H_2 for 24 h. More EtOH was added and heated to get the partly crystallized product completely into solution. Then the catalyst was filtered off. On cooling, crystals formed which were filtered off and dried to give 260 mg in two crops (80%), mp 197-199 °C, ^1H NMR (D_2O) δ 8.16 (s, 1H, H8), 6.15 (d, $J_{1',2'}$=4.6, 1H, H1'), 4.66 (app t, $J_{3',2'}$=5.4, $J_{3',4'}$=5.4, 1H, H3'), 4.57 (app t, $J_{2',1'}$=4.6, $J_{2',3'}$=5.4, 1H, H2'), 4.34 (m, 1H, H4'), 3.50 (s, 3H, OMe), 3.49 (m, 2H, H5',H5"), 2.82 (m, 1H, *CH*$(CH_3)_2$), 1.26 (s, 3H, Me), 1.24 (s, 3H, Me).

5'-N-(4-Methoxytrityl)amino-5'-deoxy-N^2-isobutyryl-2'-O-methylguanosine (10) was synthesized from **9** using 4-methoxytrityl chloride/DMAP/Et_3N/Pyr (Figure 29B) according to the procedure of Mag and Engels, *supra*, in 80% yield. ^1H NMR δ 12.11 (br s, 1H, NH), 7.95 (br s, 1H, NH), 7.70 (s, 1H, H8), 7.53-6.86 (m, 14 H, aromatic), 5.92 (d, $J_{1',2'}$=4.9, 1H, H1'), 4.55 (app t, $J_{3',4'}$=5.0, 1H, H3'), 4.35 (app t, $J_{2',1'}$=4.9, 1H, H2'), 3.84 (s, 3H, Tr-OMe), 3.55 (s, 3H, OMe), 2.82 (br s, 1H, 3'OH), 2.78 (dd, $J_{5',4'}$=3.0, $J_{5',5''}$=12.4, 1H, H5'), 2.65 (br s, 1H, NH), 2.43 (dd, $J_{5'',4'}$=5.4, $J_{5'',5'}$=12.4, 1H, H5"), 1.09 (m, 6H, 2 x Me).

5'-N-(4-Methoxytrityl)amino-5'-deoxy-N^2-isobutyryl-2'-O-methylguanosine-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (11). Using the same procedure as for the preparation of **5**, phosphoramidite **11** was obtained (Figure 29B) as a colorless foam in 80% yield after column

chromatography using 1% EtOH in CH_2Cl_2 (1% Et_3N), ^{31}P NMR δ 148.74 (s) and 148.06 (s).

Referring to Figure 29C, *3'-O-t-Butyldiphenylsilyl-N^6-benzoyl-2'-O-methyladenosine (13).* 5'-*O*-(4,4'-Dimethoxytrityl)-*N*^{6}-benzoyl-2'-*O*-methyladenosine **12** (5g, 7.3 mmol) was dissolved in DMF (20 mL) and imidazole (1.5 g, 22 mmol) and *t*-butyldiphenylsilyl chloride (2.8 mL, 10.8 mmol) were added. The mixture was stirred at rt overnight. Methanol (10 mL) was added and the solution evaporated to a syrup. After standard work up the resulting syrup was dissolved in CH_2Cl_2 (100 mL) and cooled in an ice-bath. 3% TFA in CH_2Cl_2 (v/v, 100 mL) was added and the mixture was stirred at 0 °C for 10 min. Methanol (20 mL) and toluene (50 mL) were added and the solution concentrated to a syrup *in vacuo* (40 °C). The residue was coevaporated twice with toluene and then purified by column chromatography using 1-5% MeOH in CH_2Cl_2 for elution to yield **13** as a white foam (4.3 g, 95% yield), ^1H NMR δ 8.98 (br s, 1H, NH), 8.73 (s, 1H, H2), 8.13 (s, 1H, H8), 8.02-7.39 (m, 15H, 3 x Ph), 6.06 (d, $J_{1',2'}$=7.4, 1H, H1'), 5.86 (d, $J_{OH,5'}$=10.2, 1H, 5'OH), 4.55 (m, 2H, H2',H3'), 4.20 (br s, 1H, H4'), 3.70 (d, $J_{5',5''}$=12.9, 1H, H5'), 3.14 (d, $J_{5'',5'}$=12.9, 1H, H5''), 3.10 (s, 3H, OMe), 1.15 (s, 9H, *t*-Bu).

5'-O-(4-Nitrobenzenesulfonyl)-3'-O-t-butyldiphenylsilyl-N^6-benzoyl-2'-O-methyladenosine (14) and 5'-chloro-5'deoxy-3'-O-t-butyldiphenylsilyl-N^6-benzoyl-2'-O-methyladenosine (15). (see Figure 29C). To a solution of **13** (4.3 g, 6.9 mmol) in dry pyridine (70 mL) was added 4-nitrobenzenesulfonyl chloride (2.47 g, 11 mmol) and the solution was left at rt overnight. Water (2 mL) was added and the solution concentrated to a syrup *in vacuo*. After standard work up the reaction mixture was purified by column chromatography using 1-5% gradient MeOH in CH_2Cl_2 to yield 4.7 g of the inseparable mixture of **14** and **15** in 2:1 ratio, ^1H NMR for **14** δ 8.89 (br s, 1H, NH), 8.58 (s, 1H, H2), 8.16-7.36 (m, 20H, H8, aromatic), 6.00 (d, $J_{1',2'}$=3.8, 1H, H1'), 4.56 (app t, $J_{3',4'}$=5.1, 1H, H3'), 4.33 (m, 1H, H4'), 4.27 (dd, $J_{5',4'}$=2.8, $J_{5',5''}$=11.2, 1H, H5'), 4.14 (dd, $J_{5'',4'}$=5.3, $J_{5'',5'}$=11.2, 1H, H5''), 4.09 (app t, $J_{2',1'}$=3.8, 1H, H2'), 3.20 (s, 3H, OMe), 1.11 (s, 9H, *t*-Bu), ^1H NMR for **15** δ 8.92 (br s, 1H, NH), 8.71 (s, 1H, H2), 8.16-7.36 (m, 20H, H8, aromatic), 6.15 (d, $J_{1',2'}$=3.9, 1H, H1'), 4.51 (app t, $J_{3',4'}$=5.1, 1H, H3'), 4.42 (m, 1H, H4'), 4.06 (app t, $J_{2',1'}$=3.9, 1H, H2'),

3.82 (dd, $J_{5',4'}$=4.3, $J_{5',5''}$=12.1, 1H, H5'), 3.54 (dd, $J_{5'',4'}$=3.9, $J_{5'',5'}$=12.1, 1H, H5''), 3.25 (s, 3H, *O*Me), 1.13 (s, 9H, *t*-Bu).

5'-Azido-5'-deoxy-3'-O-t-butyldiphenylsilyl-N^6-benzoyl-2'-O-methyladenosine (16). (Figure 29C) The above mixture of **14** and **15** (3.9 g) was dissolved in dry DMSO (30 mL) and LiN_3 (1.18 g, 24 mmol) was added. The reaction mixture was stirred at 80 °C overnight, then concentrated *in vacuo* (oil pump). After standard work up and column chromatography using 1-2% gradient MeOH in CH_2Cl_2 **16** was obtained as a colorless foam (2.55 g), ^{1}H NMR δ 8.92 (br s, 1H, NH), 8.72 (s, 1H, H2), 8.15 (s, 1H, H8), 8.02-7.36 (m, 15H, 3 x Ph), 6.14 (d, $J_{1',2'}$=3.4, 1H, H1'), 4.44 (app t, $J_{3',4'}$=5.1, 1H, H3'), 4.27 (m, 1H, H4'), 4.01 (app t, $J_{2',1'}$=3.4, $J_{2',3'}$=4.9, 1H, H2'), 3.53 (dd, $J_{5',4'}$=3.2, $J_{5',5''}$=13.3, 1H, H5'), 3.37 (dd, $J_{5'',4'}$=4.5, $J_{5'',5'}$=13.3, 1H, H5''), 3.29 (s, 3H, OMe), 1.13 (s, 9H, *t*-Bu).

5'-Amino-5'-deoxy-3'-O-t-butyldiphenylsilyl-N^6-benzoyl-2'-O-methyladenosine (17). Using the same procedure (Figure 29B) as for the preparation guanosine analog **9**, **16** (2.5 g, 3.9 mmol) was converted into **17** (2.25g, 94%) which resisted crystallization and was used crude in the next step, ^{1}H NMR δ 8.90 (br s, 1H, NH), 8.72 (s, 1H, H2), 8.23 (s, 1H, H8), 8.02-7.36 (m, 15H, aromatic), 6.13 (d, $J_{1',2'}$=4.4, 1H, H1'), 4.72 (app t, $J_{2',1'}$=4.4, $J_{2',3'}$=5.0, 1H, H2'), 4.17 (m, 2H, H3', H4'), 3.27 (s, 3H, *O*Me), 2.88 (dd, $J_{5',4'}$=3.2, $J_{5',5''}$ =13.8, 1H, H5'), 2.65 (dd, $J_{5'',4'}$=5.0, $J_{5'',5'}$=13.8, 1H, H5''), 1.12 (s, 9H, *t*-Bu).

5'-N-(4-Methoxytrityl)amino-5'-deoxy-3'-O-t-butyldiphenylsilyl-N^6-benzoyl-2'-O-methyladenosine (18). (Figure 29C) Using the same procedure as for the preparation of **10**, **17** was converted into **18**, which was then purified by column chromatography using 1-2% MeOH gradient in CH_2Cl_2, (2.37 g, 76%) as a colorless foam, ^{1}H NMR δ 8.90 (br s, 1H, NH), 8.02 (s, 1H, H2), 7.95 (s, 1H, H8), 8.00-6.71 (m, 29H, 3 x Ph), 6.04 (d, $J_{1',2'}$=6.4, 1H, H1'), 4.72 (app t, $J_{2',1'}$=6.4, $J_{2',3'}$=4.4, 1H, H2'), 4.65 (m, 1H, H3'), 4.33 (m, 1H, H4'), 3.80 (s, 3H, Tr-OMe), 3.20 (s, 3H, OMe), 3.03 (br s, 1H, NH), 2.26 (d, $J_{5',5''}$ =11.7, 1H, H5'), 2.15 (dd, $J_{5'',4'}$=4.3, $J_{5'',5'}$=11.7, 1H, H5''), 1.12 (s, 9H, *t*-Bu).

5'-N-(4-Methoxytrityl)amino-5'-deoxy-N^6-benzoyl-2'-O-methyladenosine (19). (Figure 29C) To the solution of **18** (2.7 g, 3 mmol) in THF (30 mL) 1 M tetrabutylammonium fluoride (TBAF) in THF (6 mL) was added and the mixture was stirred at rt 2 h. It was then concentrated to a syrup *in vacuo*. After standard work up and column chromatography using 10-30% gradient THF in CH_2Cl_2 **19** was obtained (1.6 g, 81%) as a colorless foam, ^{1}H NMR δ 8.90 (br s, 1H, NH), 8.14 (s, 1H, H2), 7.98 (s, 1H, H8), 8.02-6.79 (m, 19H, aromatic), 5.95 (d, $J_{1',2'}$=5.5, 1H, H1'), 4.91 (app t, $J_{2',1'}$=5.5, 1H, $J_{2',3'}$=5.2, H2'), 4.72 (m, 1H, H3'), 4.29 (m, 1H, H4'), 3.77 (s, 3H, Tr-OMe), 3.52 (s, 3H, OMe), 3.09 (br s, 1H, NH), 2.67 (d, $J_{OH,3'}$=3.4, 1H, OH3'), 2.60 (dd, $J_{5',5''}$ =11.7, 1H, H5'), 2.15 (dd, $J_{5'',4'}$=4.3, $J_{5'',5'}$=11.7, 1H, H5"), 1.12 (s, 9H, *t*-Bu).

5'-N-(4-Methoxytrityl)amino-5'-deoxy-N^6-benzoyl-2'-O-methyladenosine-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (20). (Figure 29C) Using the same procedure as for the preparation of **5**, **19** (1 g, 1.5 mmol) was converted into **20** and after column chromatography using CH_2Cl_2 containing 1% Et_3N (v/v) a colorless foam (0.55 g, 74%) was obtained, ^{31}P NMR δ 151.2 (s), 151.8 (s).

Referring to Figure 29D,*5'-Deoxy-5'-iodo-2'-O-methyluridine (21).* This compound was prepared from **1** using the procedure of Verheyden and Moffatt (*J. Org. Chem.*, 1970, 35, 2319, and is incorporated by reference herin in its entirety) for selective iodination of thymidine and isolated in 59% yield by column chromatography using 1-5% MeOH in CH_2Cl_2 for elution, ^{1}H NMR (DMSO-d_6) δ 7.76 (d, $J_{6,5}$=8.1, 1H, H6), 5.94 (d, $J_{1',2'}$=5.4, 1H, H1'), 5.77 (d, $J_{5,6}$=8.1, 1H, H5), 5.52 (d, $J_{OH,3'}$=6.0, 1H, 3'OH), 4.11 (dd, $J_{3',2'}$=5.36, $J_{3',4'}$=10.2, 1H, H3'), 4.06 (app t, $J_{2',1'}$=5.4, 1H, H2'), 3.93 (m,1H, H4'), 3.63 (dd, $J_{5',4'}$=5.4, $J_{5',5''}$=10.6, 1H, H5'), 3.49 (dd, $J_{5'',4'}$=6.9, $J_{5'',5'}$=10.6, 1H, H5"), 3.42 (s, 3H, OMe).

5'-(S-Triphenylmethyl)mercapto-5'-deoxy-2'-O-methyluridine (22). (Figure 29D) Sodium hydride (52 mg, 2.18 mmol) was suspended in dry DMF (1 mL) under argon at 0 °C, and a solution of triphenylmethyl mercaptan (606 mg, 2.19 mmol) in dry DMF (7 mL) was added. The mixture was stirred for 10 min at rt, cooled in ice and a solution of **21** (690 mg, 1.80 mmol) in dry DMF (5 mL) was added. After 3 h at room temperature (rt) solvent was removed *in vacuo*,

the residue dissolved in CH_2Cl_2 and washed with 5% aqueous $Na_2S_2O_3$ and water. The organic layer was dried (Na_2SO_4), evaporated to dryness and chromatographed using 1-2% MeOH in CH_2Cl_2 for elution to afford **22** (860 mg, 68%), mp 187-188 °C (EtOH-H_2O), ^1H NMR δ 8.43 (br s, 1H, NH), 7.51-7.29 (m, 16H, Tr, H6), 5.87 (d, $J_{1',2'}$=2.4, 1H, H1'), 5.78 (d, $J_{5,6}$=8.1, 1H, H5), 3.90 (m, 1H, H2'), 3.83 (m, 1H, H3'), 3.75 (dd, $J_{4',5'}$=2.4, $J_{4',3'}$=5.5, 1H, H4'), 2.81 (dd, $J_{5',4'}$=2.4, $J_{5',5''}$=13.0, 1H, H5'), 2.52 (dd, $J_{5'',4'}$=6.6, $J_{5'',5'}$=13.0, 1H, H5'').

5'-(S-Triphenylmethyl)mercapto-5'-deoxy-2'-O-methyluridine-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (23) (Figure 29D) Using the same procedure as for the preparation of **5**, 3'-phosphoramidite **23** was obtained as a white foam in 88% yield after flash chromatography purification using 50-75% gradient of EtOAc in hexane (1% Et_3N), ^{31}P NMR δ 149.1 (s) and 148.7 (s).

Ribozyme synthesis and purification

Incorporation of 5'-phosphoramidate at the *5'-end* of ribozymes. Synthesis was performed as described (Wincott *et al., supra*, incorporated by reference herin in its entirety) with a 300 s coupling time for the 5'-amino phosphoramidites **5**, **11** and **20** (Figure 29B & 29C). Detritylation was effected using a cycle that consisted of four 10 s pulses of TCA, each separated by 7 s wait steps, followed by 30 s of acetonitrile. This series was then repeated. Finally, the incoming phosphoramidite was coupled for 300 s to complete the synthesis. The ribozyme was base deprotected under standard conditions, however, desilylation was accomplished with TBAF in 24 h rather than HF/TEA solution.

Incorporation of *5'-amino group at the 5'-end.* The synthesis cycle was modified slightly from the usual protocol. The 5'-amino phosphoramidites **5**, **11** and **20** (Figure 29B & 29C) were coupled for 300 s. The usual capping reagent, acetic anhydride, was replaced with *t*-butylphenoxyacetic anhydride. All ribozymes were synthesized trityl-on. The terminal MMTr group was removed upon addition of four 10 s pulses of TCA, each separated by 7 s wait steps, followed by 30 s of acetonitrile. This series was repeated until no

orange color was observed. The ribozyme was then deprotected under standard conditions. In the synthesis incorporating **5**, a total of 323 AU of crude material resulted with 41.8% full length product (135 AU). The ribozyme was purifed by anion exchange HPLC to provide 48 AU of purified ribozyme. Similar recoveries were obtained with monomers **11** and **20**.

Incorporation of *bridging 5'-phosphorothioate at the 5'-end.* The oligomers were synthesized using the 5'-thiol phosphoramidite **23** (Figure 29D), coupled for 300 s, and the following amidite coupled for 400 s. Additionally, following the addition of the 5'-thiol amidite, capping and oxidation, the column was removed from the synthesizer. The cap and frit were removed, the support was washed out of the column and into an empty syringe with 10 mL of 200 mM $AgNO_3$ in 1:1 CH_3CN:H_2O. The syringe was capped, wrapped in foil and placed on a shaker for 1 h at rt. The mixture was then replaced into the column. The liquid was removed and the support was rinsed with 20 mL of 1:1 CH_3CN:H_2O. The support was then treated with 10 mL 50 mM DTT for 10 min at rt. The support is then washed with 20 mL H_2O, then 20 mL CH_3CN. The column was placed on the synthesizer, washed with CH_3CN for 30 s then reverse flushed for 15 s, this procedure was repeated 4 times. The synthesis was then resumed, with the next phosphoramidite coupling for 400 s and the remaining phosphoramidites coupling for the standard times.

The ribozymes were deprotected with 40% aqueous methylamine for 10 min at 65 °C. The silyl groups were removed with TEA/HF solution in 30 min at 65 °C and the oligonucleotides were precipitated from the solution. RPI.4705.5905 yielded 101.5 AU of crude material (half was lost during detritylation of 5'-STr) with 16.5% full length product.

RESULTS:

Synthesis of monomer building blocks

The key intermediates for the synthesis of ribozymes containing bridging 5'-phosphoramidate and 5'-phosphorothioate linkages were 3'-O-phosphoramidites **5**, **11**, **20** and **23** synthesized according to Figure 29.

5'-N-(4-Methoxytrityl)amino-5'-deoxy-2'-O-methyluridine monomer (5)

Uridine derivative **5** was synthesized in a way similar to that reported by Mag and Engels, *supra*, for the synthesis of a thymidine analog. 5'-Azido derivative **2** (Figure 29 B) was synthesized in one step from 2'-O-methyluridine (1) using the procedure of Yamamoto *et al., supra.* Ammonium hydroxide had to be used instead of water for the hydrolysis of intermediate 5'-phosphinimide during the conversion of **2** to **3** ((Figure 29 B)). It is well documented (Mungall *et al., J. Org. Chem.* 1975, *40*, 1659-1662) that nucleoside phosphinimines are relatively stable in water compared to simple alkyl azides. Protection of the 5'-NH_2 group of **3** with 4-methoxytrityl group, followed by standard phosphitylation afforded 3'-*O*-phosphoramidite **5** in good yield.

5'-N-(4-Methoxytrityl)amino-5'-deoxy-N^2-isobutyryl-2'-O-methylguanosine monomer (11)

Because the one-step procedure for the preparation of the 5'-azide described above does not work well for purine 2'-deoxynucleosides (Mag *et al., supra*), we used a two-step procedure for the introduction of the azido group into the 5'-position of N^2-isobutyryl-2'-*O*-methylguanosine (**6**) (Figure 29 B). Selective 5'-*O*-p-toluenesulfonation of **6** at 0 °C afforded the desired mono-substituted derivative **7** in 47% yield and 3',5'-bis-substituted derivative in 15% yield. Attempts to improve the yield and selectivity of this reaction by the portionwise addition of p-toluenesulfonyl chloride did not help. Displacement of the OTs group of **7** with an N_3 group using LiN_3 in DMSO proceeded smoothly to yield **8** in 78% yield. As in the case of uridine derivative **2** attempts to use triphenylphosphine in water/pyridine for reduction of **8** to **9** and thus avoid the simultaneous cleavage of the base labile N^2-isobutyryl group failed to hydrolyze the intermediate 5'-phosphinimine. Thus, catalytic hydrogenation of **8** using 10% Pd-C was utilized for the successful preparation of 5'-amino-5'-deoxy-2'-*O*-methyl derivative **9** (80% yield). It is worth noting that **9** underwent a gradual loss of the N^2-isobutyryl group when left in unbuffered aqueous solution for 16 h or longer. We attributed this unexpected deacylation to intramolecular base catalysis by the 5'-amino group of **9**. Protection of the free amino group of **9** with a 4-methoxytrityl

group, followed by phosphitylation afforded 3'-*O*-phosphoramidite **11** in a good yield.

5'-N-(4-Methoxytrityl)amino-5'-deoxy-N^6-benzoyl-2'-O-methyladenosine monomer (20)

The low selectivity in the tosylation of guanosine derivative **6** prompted us to to use 3'-hydroxyl protection in the preparation of adenosine analog. Thus, 5'-*O*-DMT derivative **12** was converted to 3'-*O*-TBDPSi derivative which was 5'-deprotected to yield **13** with TFA in CH_2Cl_2. The reaction of **13** with a more reactive sulfonylating agent, p-nitrobenzenesulfonyl chloride, yielded unexpectedly a 2:1 mixture of 5'-*O*-p-nitrobenzenesulfonyl and 5'-chloro-5'-deoxy substituted derivatives **14** and **15**. The mixture was treated with LiN_3 at 80 °C overnight to afford 5'-azido-5'-deoxy derivative **16** in good yield. Catalytic hydrogenation of **16** proceeded smoothly to afford 5'-amino derivative **17** which was, without purification converted to 5'-*N*-MMTr protected derivative **18**. Cleavage of the 3'-*O*-TBDPSi group was achieved using tetrabutylammonium fluoride and the resulting **19** was phosphitylated under standard conditions to give the 3'-*O*-phosphoramidite **20** in 74% yield (Figure 29C).

5'-deoxy-5'-mercapto-2'-O-methyluridine monomer (23)

Synthesis of the 5'-deoxy-5'-mercapto-2'-*O*-methyluridine monomer **23** started with selective iodination of 2'-*O*-methyluridine (**1**) using methyltriphenoxyphosphonium iodide as described (Verheyden and Moffat, *J. Org. Chem.* 1970, *35*, 2319-2326 and is incorporated by reference herin in its entirety). The iodo compound **21** was converted in 68% yield into the 5'-(*S*-triphenylmethyl)mercapto compound **22** using the sodium salt of triphenylmethyl mercaptan in DMF as described by Sproat *et al.*, (*Nucleic Acids Res.* 1987, *15*, 4837-4848 and is incorporated by reference herin in its entirety). Introduction of an aqueous $Na_2S_2O_3$ wash into the work up step was beneficial in reducing the cleavage of *S*Tr group and formation of intermolecular disulfide bonds by any iodine present in the reaction mixture (Kamber, *Helv. Chim. Acta* 1971, *54*, 398-422) Phosphitylation of **22** under standard conditions (Atkinson, T., Smith, M. In *Oligonucleotide Synthesis: A*

Practical Approach, Gait, M.J., Ed.; IRL Press: Oxford, 1984, pp 35-81, and is incorporated by reference herin in its entirety) yielded 3'-*O*-phosphoramidite **23** (Figure 29 D).

Oligonucleotide synthesis

Synthesis of oligomers with *bridging* 5'-Phosphoramidate

There are four issues that must be addressed when synthesizing oligomers containing bridging 5'-phosphoramidate linkages:

1. Coupling of the 5'-amine containing phosphoramidite to the growing chain; 2. Coupling of the following amidite to the 5'-amine; 3. Deprotection conditions; 4. Removal of the MMT protecting group from the 5'-amine.

After an extensive study on incorporation of 5'-amino modified monomers into ribozymes (see Table VI), we found that a coupling time of 300 s for **5** and 300 s for the following 2'-*O*-Me nucleotide provided the best results. For optimal results, the oligomer was desilylated with TBAF rather then HF/TEA solution as more full length polymer was produced with the former reagent.

We devised an experiment to study the influence of extended exposure of the modified oligonucleotides to the detritylation solution (TCA/CH_2Cl_2) and activator (tetrazole). Following completion of the synthesis, we exposed one oligomer to four "dummy cycles" of detritylation solution and another to four "dummy cycles" of activator. Although no impact upon full length product was observed with the extended detritylation exposure, there did appear to be a detrimental effect to extended exposure to activator.

Finally we investigated the removal of the MMT protecting group. The optimal procedure for removal of the MMT group required a "flow through" process. Therefore, detritylation was effected using four 10 s pulses of TCA with 7 s wait steps between each pulse. This was followed by 30 s of acetonitrile and then the four 10 s pulses of TCA were repeated. The incoming amidite was then coupled for 300 s to complete the synthesis.

Synthesis of oligomers with *5'-amino group at the 5'-end:*

In the process of synthesizing ribozymes containing phosphoramidate linkages at the 5'-end, we also synthesized ribozymes that contained 5'-amines at the 5'-terminus of the ribozyme. The standard synthetic protocols were modified slightly to optimize synthesis. To ensure complete removal of the more stable MMTr protecting group on the 5'-amine, the final detritylation step was adjusted as in the previous example. In addition, t-butylphenoxyacetic anhydride was used as the capping reagent. We had observed the formation of a side product, identified by MALDI-TOF MS as the *N*-acetylated ribozyme, when acetic anhydride was the capping agent.

Synthesis of oligomers with *bridging 5'-phosphorothioates:*

A single bridging 5'-phosphorothioate linkage was incorporated into the 5'-end of two ribozymes. The 5'-thiol phosphoramidite **23** was coupled for 300 s and the following phosphoramidite coupled for 400 s. The ribozymes were base deprotected as usual and then treated with TEA/HF at 65 °C for 0.5 h rather than 1.5 h. Using the latter reagent we have not observed substantial cleavage of the P-S bond as observed when TBAF was used (Sund *et al., supra*).

Ribozymes containing 5'-amine at the 5'-end showed resistance to digestion by calf spleen 5'-exonuclease equivalent to that observed with P=S backbone modifications. Also, their catalytic activity was comparable to the wild type ribozymes as described *infra.*

Example 16: Nuclease stability, *in vitro* activity and cell culture efficacy of 5'-amino-modified ribozymes

Materials and Methods:

Radio-labeling of Ribozymes and Substrates. Ribozymes and substrates were 5'-end-labeled using T4 Polynucleotide Kinase and γ-^{32}P-ATP. For internal labeling, ribozymes were synthesized in two halves with the junction 5' to the GAAA sequence in Loop II (Figure 27). The 3'-half-ribozyme portion was 5'-end-labeled using T4 Polynucleotide Kinase and γ-^{32}P-ATP, and was then ligated to the 5'-half-ribozyme portion using T4 RNA ligase. Labeled

ribozymes were isolated from half-ribozymes and unincorporated label by gel electrophoresis.

Ribozyme Activity Assay. Ribozymes and 5'-^{32}P-end-labeled substrate were heated separately in reaction buffer (50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$) to 95 °C for 2 min, quenched on ice, and equilibrated to the final reaction temperature (37 °C) prior to starting the reactions. Reactions were carried out in enzyme excess, and were started by mixing ~1 nM substrate with the indicated amounts of ribozyme (50 nM-1 µM) to a final volume of 50 µL. Aliquots of 5 µL were removed at 1, 5, 15, 30, 60 and 120 min, quenched in formamide loading buffer, and loaded onto 15% polyacrylamide/8 M Urea gels. The fraction of substrate and product present at each time point was determined by quantitation of scanned images from a Molecular Dynamics PhosphorImager. Ribozyme cleavage rates were calculated from plots of the fraction of substrate remaining *vs* time using a double exponential curve fit (Kaleidagraph, Synergy Software). The fast portion of the curve was generally 60-90% of the total reaction, so that observed cleavage rates (k_{obs}) were taken from fits of the first exponential.

Enzymes. Calf Spleen 5'-exonuclease was purchased from Boehringer Mannheim. T4 polynucleotide kinase and Lambda 5'-exonuclease were purchased from GIBCO/BRL. Enzyme reactions were performed according to the manufacturers' suggestions.

Cell Culture. Rat aortic smooth muscle cells (SMC) were isolated from aortic tissue explants from 69-84 day-old female Sprague-Dawley rats (Harlan Sprague Dawley, Inc.) and assayed through passage six. SMC were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with nonessential amino acids (0.1 mM of each amino acid), 0.1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 20 mM HEPES (all from BioWhittaker) and 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc.).

Preparation of Smooth Muscle Cell Extracts. Rat smooth muscle cell nuclear or total cell extracts were prepared by harvesting SMC from 3 confluent T150 flasks. For nuclear lysates, SMC were trypsinized from the

flasks, washed twice with PBS, and resuspended in 500 µL of hypotonic buffer. After 40 strokes with a Dounce B homogenizer, 300 µL of 34% sucrose was added and nuclei were pelleted by centrifugation at 4 °C and 500 x g for 10 min. The nuclei were washed with a solution containing 500 µL of hypotonic buffer and 300 µL of 34% sucrose, then repelleted. The pellet was resuspended in buffer A (10 mM Tris-HCl, pH 7.5; 400 mM NaCl; 1.5 mM $MgCl_2$, 0.1 mM EGTA, 5% glycerol, 0.5 mM DTT, and 0.5 mM PMSF) and given 20 strokes in the Dounce B homogenizer. The resultant suspension was gently shaken for 30 min at 4 °C and then dialyzed at 4 °C for 4 h against 100 mL of dialysis buffer (20 mM Tris-HCl, pH 7.5; 0.1 mM EDTA, 75 mM NaCl, 20% glycerol, 0.5 mM DTT and 0.5 mM PMSF). After dialysis, the solution was centrifuged at 4 °C and 16000 x g for 30 min. Aliquots of the supernatant were frozen on dry ice and stored at -70 °C. Separate aliquots were used for each assay.

Total cell lysates were prepared by rinsing trypsinized cell preparations 3 x in PBS and pelleting by centrifugation. The pellets were resuspended in 1 mL of DMEM, 0.5 mM PMSF. PMSF was added as a precaution to minimize proteolytic activity during isolation. Cells were freeze-thawed 3 times and disrupted by 40 strokes in a Dounce B homogenizer. Aliquots of whole cell lysates were aliquoted and frozen at -70°C. Separate aliquots were used for each assay.

Ribozyme Stability Assay. One half pmol of gel-purified, internally labeled ribozyme was added to 20 µL of reaction buffer (67 mM glycine-KOH [pH 9.4], 2.5 mM $MgCl_2$, and 50 µg/mL BSA; containing either 1 µL of calf spleen 5'-exonuclease [2U/2 mg/mL] or 10 µL of smooth muscle cell lysate). Samples were placed at 37 °C and 3 µL aliquots were withdrawn at 0, 30, 60, 120 and 240 min, and 24 h.. Aliquots were quenched by the addition of 12 µL of 95% formamide, 0.5 x TBE (50 mM Tris, 50 mM Borate, 1 mM EDTA) and were frozen prior to gel loading. Ribozyme integrity was assessed using electrophoresis in 12% acrylamide/7M urea gels. Undigested ribozyme samples were used as size controls. Gels were imaged by autoradiography.

Proliferation Assays. Cells were plated in growth medium in 24-well plates at $5x10^3$ cells per well. After 24 hours, the medium was removed, cells

were washed twice with PBS containing Ca^{2+}/Mg^{2+}, and starvation medium was added. Starvation medium is growth medium in which the concentration of FBS is reduced to 0.5%. Cells were starved for 68-72 hours before ribozyme treatment. Ribozymes were diluted in serum-free DMEM with additives as above excluding antibiotics. LipofectAMINE (Gibco-BRL) was added to a final concentration of 3.6 µM DOSPA (= 7.2 µg/mL LipofectAMINE). Lipid/ribozyme mixtures were vortexed, incubated for 15 minutes, and then added to cells which had been washed twice with PBS containing Ca^{2+}/Mg^{2+}. Cells were incubated with the ribozyme/lipid complexes at 37 °C for 4 hours before the mixture was aspirated away. Cells were stimulated by the addition of growth medium. Control cells were treated with lipid only and stimulated with growth medium containing either 10% or 0% FBS. All conditions were run in triplicate. At the time of stimulation, 5'-bromo-2'-deoxyuridine (BrdU, Sigma) was added at a final concentration of 10 µM. Cells were incubated for 24 h and then fixed by the addition of cold 100% methanol plus 0.3% hydrogen peroxide for 30 min at 4 °C. The following reagents were used at room temperature, unless otherwise noted, to stain the BrdU containing nuclei: i) 2 M HCl for 20 minutes; ii) 1% horse serum in PBS overnight at 4 °C; iii) anti-BrdU monoclonal antibody (Becton-Dickinson) diluted 1:200 in 1% bovine serum albumin and 0.5% Tween 20 for 1 hour; iv) biotinylated horse anti-mouse IgG in DPBS for 30 minutes; v) ABC Reagent (Pierce mouse IgG kit) in DPBS for 40 minutes; vi) DAB substrate (Pierce) diluted 1:10 in DAB buffer (Pierce) for 7-10 minutes; and vii) hemotoxylin (Fisher) diluted 1:1 in deionized water for 1-2 minutes. A minimum of 500 cells per well were counted under the microscope and the percentage of proliferating cells (BrdU-stained nuclei/total nuclei) was determined.

Resistance of 5'-amino-modified ribozymes to digestion by calf spleen 5'-exonuclease.

Internally-labeled ribozymes were prepared by the separate synthesis of 5'-and 3'-half ribozymes, ^{32}P end-labelling of the 3'-half ribozyme at the 5'-terminus and subsequent ligation of appropriate 5'- and 3'-half ribozymes to produce a full-length ribozyme with an internal ^{32}P label. For stabilization against digestion by 3'-exonuclease, the 3'-ends of all ribozymes were capped with a 3'-3' linked abasic residue (Figure 32B; Beigelman *et al.*, 1995

supra). Unless otherwise noted, nonessential residues contained 2'-*O*-Me modifications, while essential residues contained 2'-ribose moieties as illustrated in Figure 27. Modifications to ribozymes at positions 2.1-2.7 and substitutions at positions U4 and U7 are summarized in Table III. While ribozymes containing either ribose (**Rz 1**) or deoxyribose (**Rz 2**) moieties at positions 2.1-2.7 were rapidly digested by calf spleen 5'-exonuclease, ribose containing ribozymes appeared to be more resistant to digestion. 2'-*O*-Me modification at positions 2.1-2.7 (**Rz 3**) slowed digestion but did not prevent nucleolytic loss of the Stem I region after extended incubation with calf spleen exonuclease. Analysis of the digestion patterns revealed that progressive exonucleolytic digestion within each of these ribozymes stopped near the U4-amino modified residues. Identification of the U4 position as the limiting site for exonuclease digestion was achieved by counting down the digestion ladders of **Rzs 1** and **2** on a gel.

Ribozymes containing partial P=S backbone (positions 2.1-2.7, **Rz 4**) or 5'-amino (**Rz 6**) modifications were resistant to digestion by exonuclease even after a 24 h incubation with the calf spleen enzyme. Although the data discussed used ribozymes containing U4/U7 amino substitutions, we found that U4-*C*-allyl modified ribozymes with similar P=S or 5'-amino modifications were also stable to 5'-exonucleolytic attack (*e.g.*, **Rz 8**). A low level of contaminating endonuclease activity was observed in these assays and accounts for the decreased amounts of full-length P=S or 5'-amino modified ribozymes after 24 h of incubation. Similar patterns of nuclease resistance were observed for these ribozymes in parallel assays using Lambda 5'-exonuclease.

Ribozyme stability in rat smooth muscle cell lysates.

Internally-labeled ribozymes were prepared for lysate stability assays as described in the previous section and in Materials and Methods. The 3'-ends of all ribozymes contained a 3'-3' linked abasic residue. Ribose and 2'-*O*-Me substitutions into the ribozyme used standard patterns which were discussed above. Modifications to positions 2.1-2.7 and 5'-end substitution for the ribozymes are summarized in Table III. The data show that ribozymes containing unprotected ribose (**Rz 1**) or deoxyribose (**Rz 2**) residues in

positions 2.1-2.7 are digested in both nuclear and whole cell lysates, but at a much slower rate than was observed in assays containing purified calf spleen 5'-exonuclease. Incubation of these ribozymes in SMC lysates resulted in the progressive shortening of ribozyme fragments over time, suggesting that the molecules were being digested by a cellular 5'-exonuclease activity. While progressive 3'-end digestion by an uncharacterized cellular enzyme cannot be ruled out in these assays, previous results in serum and cell extracts have shown that the addition of a 3'-3' abasic residue at the 3'-terminus renders ribozymes resistant to 3'-exonucleolytic attack (Beigelman *et al.*, 1995 *supra*).

Neither 2'-*O*-Me (**Rz 3**), P=S backbone (**Rz 4**) or 5'-amino (**Rzs 6** and **8**) modification of ribozymes totally protected the molecules from digestion in SMC extracts. An examination of the digestion patterns revealed that while there was no exonucleolytic cleavage of these ribozymes, they were fragmented by endonucleolytic attack. 2'-substitution for the U4/U7-amino groups of **Rz 6** using U4/U7-*C*-allyl/*O*-Me groups of **Rz 8** did not affect the resistance of 5'-amino containing ribozymes to exonucleolytic attack. Taken together with the data from the previous section, these data show that while 2'-*O*-Me modification can provide limited protection against 5'-exonucleolytic digestion in cellular extracts, 2'-*O*-Me substitution provides much less protection versus digestion by purified 5'-exonuclease. In contrast, P=S backbone and 5'-amino modifications prevented digestion by both purified calf spleen 5'-exonuclease and SMC 5'-exonuclease(s) but provided little added protection from endonucleolytic attack at the essential ribose residues (positions 5, 6, 8, 12 and 15.1).. Based on these data and previous reports of the ability of U4/U7 modifications to restrict endonucleolytic attack at essential ribose residues (Beigelman *et al.*, 1995 *supra*), we conclude that the effects of P=S and 5'-amino substitutions are confined to a very localized region at the 5'-end of the ribozyme.

The digestion profiles of ribozymes containing ribose (**Rz 1**) or deoxyribose (**Rz 2**) residues at positions 2.1-2.7 were quite different in the two SMC lysates. Although there was approximately 10 times more protein in the cellular lysates than in the nuclear lysates, this alone cannot account for the differences, because the degree of digestion for **Rz 2** in cellular lysates was more than 10 x greater than greater in nuclear lysates. In contrast, the degree

of digestion for **Rz 1** was approximately the same in both lysates at all times. These data suggest that nucleolytic digestion of ribozymes in SMC lysates is highly dependent upon the chemical nature of the ribozymes. Differences in the digestion patterns of **Rz 1** and **Rz 2** suggest that different enzymes may be responsible for the exonucleolytic digestion near the 5'-regions of these molecules. This differential chemical susceptibility of ribozymes to nucleolytic digestion was even more obvious when other cell lysates were used for comparison and in some cases (*e.g.*, HL60 cell lysates) the ribose-containing Stem I regions were more susceptible to digestion than the deoxyribose-containing stems. Such comparative data show that the susceptibility of ribozymes to digestion by cellular nucleases is highly dependent upon both cell type and chemical modification to the ribozyme.

On the basis of the nuclease assays, we conclude that 1) 5'-amino modified ribozymes are as resistant to 5'-exonucleolytic digestion as thioated ribozymes, and 2) the advantage which P=S modifications give to ribozyme efficacy in cells is not just a result of their superior nuclease stability, but probably also results from intracellular localization or protein association which is mediated by the thioate moieties within the ribozymes.

Catalytic activity of 5'-amino modified ribozymes.

The relative effect of 5'-amino substitution on ribozyme catalytic activity was investigated under standard assay conditions as described, *supra*, in Materials and Methods. The catalytic activity of each ribozyme was assayed at two concentrations and the results were plotted to determine the region of the reaction which gave exponential rates at each concentration. Cleavage rates (k_{obs} values) were calculated from fits of the first exponential. Table IV shows an activity comparison for the five U4/U7-amino containing ribozymes at concentrations of 40 and 500 nM (roughly 4 and 50 fold above K_M). Activity is presented both as the cleavage rate (min^{-1}) and as a percentage of the rate for the control, **Rz 3**.

Comparison of the catalytic rates of selected ribozymes from Table III revealed that neither P=S nor 5'-amino modification of **Rz 3** (**Rzs 4** and **6**, respectively) affected the catalytic rate significantly. Ribozymes containing 2'-

O-Me substitutions at positions 2.1-2.7 (**Rz 3**) (Figure 27) showed slightly better catalytic activity (20- 30%) in this assay than ribozymes containing ribose moieties at these positions (**Rz 1**). As reported earlier (Beigelman *et al.*, 1995 *supra*), we generally see very similar catalytic rates for ribozymes containing ribose and 2'-*O*-Me substitutions at positions 2.1-2.7 (Figure 27) although there are generally also substitutions at positions 15.1 -15.7 (Figure 27) in the molecules which have been compared. The k_{obs} values for P=S and 5'-amino modified ribozymes (**Rz 4** and **6**, respectively) were equivalent, within error, to those of the ribose-containing **Rz 1**.

The deoxyribose-substituted **Rz 2** is peculiar in that it showed a 6-10 fold reduction in activity when compared with the other 2.1-2.7 position (Figure 27) substitutions (**Rzs 1, 3, 4, and 6**). The similarity in cleavage rates at 40 and 500 nM for this ribozyme suggest that the reduced k_{obs} for **Rz 2** was not a result of reduced binding affinity but more likely reflects a 6-10 fold decrease in k_{cat}.

This data represents the first comparative report of the effects of substitution at positions 2.1-2.7 into ribozymes using U4/U7-amino (or U4/N7-amino) stabilized ribozymes and additionally demonstrates that nuclease stabilizing modifications can be used to replace P=S backbone substitutions in ribozymes without reducing catalytic activity.

Cellular efficacy of 5'-amino modified ribozymes.

Based on catalytic data (Table IV) and the increased stability observed with 5'-amino modified **Rz 6** and **8** in the nuclease assays, we decided to compare the efficacy of **Rz 6** to the thioated **Rz 4** in cell assays of ribozyme activity. The relative abilities of ribozymes containing various modifications at positions 2.1-2.7 (Figure 27) and/or the 5'-terminus were compared in a cell proliferation assay using rat smooth muscle cells. Ribozymes were delivered using lipofectAMINE as described, *supra*, in the Materials and Methods section. After the application of ribozymes, cells were metabolically labeled with BrdU for 24 h and the number of proliferating SMC nuclei were determined by differential staining using an anti-BrdU antibody detection system and hematoxylin.

Ribozymes containing ribose (**Rz 1**), deoxyribose (**Rz 2**) or 2'-*O*-Me modified (**Rz 3**) nucleosides at positions 2.1-2.7 as well as catalytically inactive (**Rzs 5, 7, 9, and 11**) were included as controls for non-specific ribozyme inhibition. The stability data suggested that **Rz 1** and **2** would be unstable in SMC, and previous results comparing thioated and nonthioated ribozymes suggested that even though **Rz 3** is relatively nuclease-resistant in the SMC lysates, nonthioated ribozymes would be less effective in cellular assays. Ribozymes with catalytically inactivated core regions (**Rz 5, 7, 9, and 11**) were included to differentiate true ribozyme activity from non-specific phosphorothioate effects. Ribozymes with catalytically active cores containing either U4/U7-amino or U4/U7-C-allyl-*O*-Me modifications and P=S (**Rz 4** and **Rz 10**, respectively) or 5'-amino modifications (**Rz 6** and **Rz 8**, respectively) were included as positive controls. The relative abilities of each ribozyme to inhibit SMC proliferation are summarized in Table VII and shown graphically in Figures 30 and 31.

As shown in Table VII, ribozymes with ribose (**Rz 1**), deoxyribose (**Rz 2**) or 2'-*O*-Me (**Rz 3**) moieties at positions 2.1-2.7 exhibited similarly low levels of inhibitory activity in the SMC proliferation assay. The deficiency of inhibitory action by either **Rz 1** or **Rz 2** reflected the inherent nuclease susceptibility of these molecules in SMC lysates and suggested that even the low levels of nuclease activity which we observed in the lysates may be enough to digest unstablized ribozymes quickly within the cellular enivironment. Alternatively, **Rzs 1** and **2** may be showing lower efficiency of inhibition of cellular proliferation because they are not localized near target molecules. The lower efficacy with **Rz 3** is consistent with this latter hypothesis. Based upon our data showing the resistance of **Rz 3** to digestion using purified preparations of calf spleen 5'-exonuclease, these molecules are expected to be relatively stable within the cells, yet they don't decrease cellular proliferative rates any better than **Rzs 1** or **2**. We feel that **Rz 3** preparations are stable within cells and the decreased inhibitory activity may be because of issues unrelated to their nuclease susceptibility.

Comparison of the relative efficacies showed that U4/U7-amino containing 5'-amino-modified **Rz 6** was as effective at inhibiting SMC proliferation as the thioate-stabilized **Rz 4**. Both of these molecules were

more effective than the 5'-amino, U4-*C*-allyl modified **Rz 8**, which was slightly more active than **Rzs 1-3**. Further, **Rz 6**, but not **Rz 4** showed better efficacy than their catalytically inactive counterparts, **Rzs 7** and **5**, respectively. These data show that P=S modifications of ribozymes enhance their cellular efficacy over that seen with non-stabilized ribozymes. Similar efficacies can be achieved without the apparent non-specific effects of the thioated compounds when other nuclease-stabilizing chemistries are present within the ribozyme structure (*e.g.*, the 5'-amino modification). The further observation that nuclease-stable, **Rz 5** exhibited better inhibitory activity than nuclease-sensitive, catalytically active **Rzs 1** and **2** shows that nuclease stabilization is important for efficient ribozyme efficacy in cells when the ribozymes are delivered exogenously..

In summary, we have found that 5'-amino, U4/U7-amino modified ribozymes exhibited in vitro stability, *in vitro* catalytic activity and cellular efficacy (Figure 30 and 31) which was equivalent to similar thioated, U4/U7-amino modified ribozymes. Additionally, 5'-amino containing ribozymes showed slightly better cellular efficacy when using the U4/U7-amino format (Figure 30, 5'-amino Active RZ) than with the U4-*C*-allyl format (Figure 31, 5'-amino Active RZ). This latter observation reflected slightly better *in vitro* catalytic activities which were observed with the U4/U7-amino compounds.

Taken together, these data support the notion that a 5'-amino modification to ribozymes will enhance their intracellular stability and enable intracellular efficacy in a manner which is consistent with their observed relative catalytic rates *in vitro*. Although it is not possible to determine on the basis of these studies whether 5'-amino containing ribozymes colocalize to the same intracellular region as thioated ribozymes, these results do suggest that 5'-amino modified ribozymes can be used effectively in animal studies of ribozyme efficacy without exhibiting some of the concentration dependent non-specific effects which have been observed by others when using thioated antisense oligonucleotides.

Example 17: Terminal Modification of Ribozymes Using Phosphorothioates

Comparison of 5'-end versus 3'-end modifications- Ribozymes targeting c-*myb* site 575, as described in Example in 3, *supra*, were complexed with LipofectAMINE and delivered to rat aortic smooth muscle cells at a 100 nM dose. Cell proliferation was measured as described in Materials and Methods of Example 3, *supra*. Active and inactive versions of several different chemical modifications were tested. "2'-*O*-Me" indicates an RNA core with five 2'-*O*-methyl residues at the 5'- and 3'-ends. "2'-*O*-Me P=S" indicates an RNA core with five 2'-*O*-methyl phosphorothioate residues at the 5'- and 3'-ends. "U4 *C*-allyl" and "U4 *C*-allyl P=S" indicate U4 2'-*C*-allyl "stabilized" cores without and with phosphorothioate linkages at the 5'- and 3'-ends, respectively. "U4,7 NH_2" and "U4,7 NH_2 P=S" indicate U4 and U7 2'-amino "stabilized" cores without and with phosphorothioate linkages at the 5'- and 3'-ends, respectively. Relative smooth muscle cell proliferation is calculated as follows: (%proliferation with ribozyme - %basal proliferation) ÷ (%proliferation with serum - %basal proliferation) x 100.

The results indicate that both a nuclease-resistant core and phosphorothioate linkages in the binding arms are necessary for significant cell culture efficacy when the ribozymes are delivered exogenously. Since phosphorothioate linkages may be associated with some degree of cytotoxicity and some non-specific effects [Uhlmann *et al.*, 1990 *Chem. Rev.* 90, 543], we wished to determine the minimum number of phosphorothioates sufficient for ribozyme-mediated cell efficacy. A comparison of ribozymes containing either 5 phosphorothioate linkages at the 5'-end, or 5 phosphorothioate linkages at the 3'-end, or 5 phosphorothioate linkages at both the 5'- and 3'-ends. The ribozyme containing phosphorothioates only at the 3'-end showed only marginal efficacy when compared with an inactive ribozyme, while the ribozyme containing phosphorothioates at the 5'-end showed equivalent efficacy to that containing phosphorothioates at both the 5'- and 3'-ends. In this experiment, the inactive ribozyme showed some inhibition relative to the vehicle-treated control. A ribozyme with scrambled sequence binding arms exhibited an equivalent degree of inhibition to an inactive ribozyme, indicating that this effect was not mediated by ribozyme binding, but was truly a "non-specific" effect on proliferation. Next, we compared

ribozymes with varying numbers of phosphorothioates at the 5'-end. The degree of efficacy gradually decreased as the number of phosphorothioate linkages was reduced. From these experiments we concluded that a minimum of four to five phosphorothioate linkages at the 5'-end is sufficient to maintain optimal efficacy.

The ribozymes used in this study contained either 3'-phosphorothioate linkages, or a 3'-3' "inverted thymidine" modification to protect against 3'-exonuclease activity. We have subsequently shown that the outcome of this assay is not particularly sensitive to the presence or absence of this 3'-protecting group. C-*myb* ribozymes containing various protecting groups including a 3'-3' inverted thymidine, a 3'-3' inverted abasic residue, a 3'-butanediol showed equivalent efficacy in inhibiting smooth muscle cell proliferation .

Example 18: Incorporation of phosphorodithioate linkages into ribozymes

Materials and methods

Referring to Figure 33, 2'-O-TBDMS-5'-O-DMT-N-protected ribonucleosides, 5'-O-DMT-N-protected deoxy- and 2'-O-Me ribonucleosides were from Chem Genes Corporation, Waltham, MA. Commercially available anhydrous solvents were employed without purification. Concentrations of solutions were carried out in vacuo at 40 °C or lower using an aspirator or an oil vacuum pump. Solids were dried at room temperature in a desiccator over phosphorus pentoxide and potassium hydroxide. ^{31}P NMR spectra were recorded on a Varian Gemini 400 spectrometer operating at 161.947 MHz with 85% phosphoric acid as external standard. Oligonucleotides were synthesized on an Applied Biosystems 381A synthesizer using Applied Biosystems columns.

General procedures

Ribonucleoside 3'-S-(2-cyanoethyl)N,N-dimethylthiophosphoramidite synthesis:

Suitably protected 2'-*t*-butyldimethylsilyl-5-O'-dimethoxytrityl nucleoside (2.0 mmol) (Figure 33) was dried and was dissolved in dry dichloromethane (CH_2Cl_2) (20 ml) under argon and the solution was cooled to 0 °C (ice-bath). The mixture of N,N-diisopropylethylamine (DIPEA) (0.56 ml, 3.20 mmol) and N,N,N',N'-tetramethylchlorophosphordiamidite [$PCl(NMe_2)_2$] (0.40 g, 2.60 mmol) in dry CH_2Cl_2 (5 ml) was added dropwise to the above solution under constant stirring. The mixture was stirred at rt for 30 min after which time β-mercaptopropionitrile (0.44 g, 5.0 mmol) was added and the reaction mixture was stirred at rt for additional 1 h. The mixture was then poured into CH_2Cl_2 (100 ml, 1% triethylamine) and washed with saturated $NaHCO_3$ (100 ml), 10% aq. Na_2CO_3 (2 x 100 ml) and saturated brine (100 ml). The organic layer to which 1 ml of Et_3N was added was dried (Na_2SO_4) for 20 min and concentrated to ca 10 ml in vacuo. This solution was added dropwise into the stirred , cooled (0 °C), degassed hexanes (200 ml, 1% Et_3N). The precipitate was filtered off and dried in vacuo to yield the product as a white powder.

2'-Deoxy- and 2'-O-methylribonucleoside 3'-S-(2-cyanoethyl)N,N-dimethylthiophosphoramidite synthesis:

Suitably protected 5'-O-dimethoxytrityl nucleoside (4 mmol) and DIPEA (1.05 ml, 6.0 mmol) were dried and were dissolved in dry CH_2Cl_2 (30 ml) under Ar and the solution was cooled to 0 °C (ice-bath). $PCl(NMe_2)_2$ (0.62 g, 4.0 mmol) was added dropwise under stirring. The clear solution was stirred at rt for 10 min, then β-mercaptopropionitrile (0.42 g, 4.8 mmol) was added and the solution was stirred at rt for additional 1 h. The work up of the reaction mixture as described for ribonucleosides above yielded products as white powders.

Synthesis with manual thiolation:

Model syntheses of ribo and 2'-*O*-methyl dithioate oligonucleotide sequences was performed on an ABI model 394 synthesizer using a modified synthesis cycle for thiolation. A 10 µmol cycle was created to accomodate manual sulfurization off of the instrument. This was accomplished by placing an interrupt step immediately after the phosphoramidite coupling step following the final acetonitrile wash and argon flush. The synthesizer column

containing the oligo bound solid support was subsequently removed from the instrument. One frit was then removed from the end of the column and a 20 ml syringe attached to that end. At the other end of the column (the end with a frit) was attached a 20 ml syringe containing a solution of 1.5 g elemental sulfur dissolved in 20 ml of carbon disulfide and 2,6-lutidine (1:1 by volume). By forcing the thiolation solution through the column, the support was transferred to the empty syringe. This syringe, now containing the support suspended in thiolation solution, was capped off and placed on an orbital shaker for one hour. The syringe containing the suspended support was then reattached to the end of the column without a frit and the contents transferred back to the column. A new frit was then placed on the column. Excess sulfur was then washed off the support with a 20 ml solution of carbon disulfide/2,6-lutidine 1:1 followed by 20 ml anhydrous acetonitrile. Synthesis was then resumed by placing the column back on the instrument. The synthesizer cycle was resumed and the entire process repeated as necessary for each dithioate substitution introduced into the oligo. It should be noted that a 300 second coupling time was utilized for 2'-*O*-methyl residues while a 600 second coupling time was utilized for ribo residues. Also, the use of S-ethyl tetrazole was avoided in order to minimize side reactions resulting from the more labile dimethylamino substituted phosphoramidite moiety. Also note oxidation prior to capping in the cycle. Cleavage from the support and deprotection results from treatment of the solid support with a solution of 15% benzene or toluene in saturated ethanolic ammonia (-70°C sat.) for 2 hours at rt and 15 hours at 55°C. Our studies demonstrate 90% thiolation efficiency under these conditions as determined by 31P NMR analysis of crude material.

Synthesis with automated thiolation:

A new synthesizer cycle (2.5 µmol) was created for fully automated synthesis of 2'-*O*-methyl and ribo phophorodithioate oligonucleotides. Tetrazole was used in place of S-ethyl tetrazole to minimize side reactions. The following bottle positions on the ABI 394 synthesizer were assigned to the following solutions:

position #10: carbon disulfide:pyridine:TEA, elemental sulfur (95:95:10, 5%) Note: this solution must be used within 24 hours for optimum results.

position #15: carbon disulfide

position #19: dichloromethane

The synthesis cycle was designed to deliver 12 equivalents or less of phosphoramidite with 600 second coupling times for ribo residues and 300 second coupling times for 2'-*O*-methyl residues. After coupling, thiolation solution (bottle #10) is delivered in two pulses. In our studies, the thiolation time was varied between 1 and 60 minutes, with an optimum time of 6 minutes. Care must be taken to avoid precipitation of sulfur in the synthesizer lines; as such, carbon disulfide (bottle #15) washes precede and follow delivery of the thiolation solution. Dichloromethane washes (bottle #19) are used to remove excess carbon disulfide from the column. In our studies, oxidation with aqueous iodine/pyridine followed standard capping in order to visualize incomplete thiolation by 31P NMR. This step was necessary for optimization, but is to be removed from standard synthetic dithioate protocols due to the increased possibility of phosphorothioate and phosphodiester contamination. Cleavage from the support and deprotection results from treatment of the solid support with a solution of 15% benzene or toluene in saturated ethanolic ammonia (-70°C sat.) for 2 hours at rt and 15 hours at 55°C. Our studies demonstrate 90% thiolation efficiency under these conditions as determined by 31P NMR analysis of crude material.

Example 19: General procedure for the synthesis of carbocyclic nucleoside Phosphoramidites

Referring to Figure 35, carbocyclic nucleosides (1) are synthesized essentially as described by Agrofoglio *et al.*, 1994, *Tetrahedron* 50, 10611. Carbocyclic nucleosides (1) were 5'-protected for example by 5'-*O*-dimethoxytritylating **1** according to the standard procedure (see Oligonucleotide Synthesis: A Practical Approach, M.J. Gait Ed.; IRL Press, Oxford, 1984, p 27, and is incorporated by reference herin in its entirety) to yield **2** in high yield in the form of yellowish foams after silica gel column chromatography. To the stirred solution of the protected nucleoside **2** in 50 mL of dry THF and pyridine (4 eq), $AgNO_3$ (2.4 eq) was added. After 10 minutes tert-butyldimethylsilyl chloride (1.5 eq) was added and the reaction mixture was stirred at room temperature for 12 hours. The

resulted suspension was filtered into 100 mL of 5% aq $NaHCO_3$. The solution was extracted with dichloromethane (2x100 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 and evaporated. The residue containing **3** was purified by flash chromatography on silica gel. Compound **3** was then phosphitylated in the following way: To the ice-cooled stirred solution of protected nucleoside **3** (1 mmol) in dry dichloromethane (20 mL) under argon blanket was added dropwise via syringe the premixed solution of N,N-diisopropylethylamine (2.5eq) and 2-cyanoethyl N',N- diisopropylchlorophosphoramidite (1.2 eq) in dichloromethane (3 mL). Simultaneously via another syringe N-methylimidazole (1 eq) was added and stirring was continued for 2 hours at room temperature. After that the reaction mixture was again ice-cooled and quenched with 15 ml of dry methanol. After 5 min stirring, the mixture was concentrated in vacuo (<40°C) and purified by flash chromatography on silica gel to give corresponding phosphoroamidite **4**.

Carbocyclic nucleoside phosphoramidites are incorporated into ribozymes using solid phase synthesis as described by Wincott *et al.*, 1995 *supra*, incorporated by reference herein in its entirety. The ribozymes are deprotected using the standard protocol described above.

Example 20: General procedure for the synthesis of alpha nucleoside Phosphoramidites

Referring to Figure 36, alpha nucleosides (**1**) are synthesized essentially as described by Debart *et al.*, 1992, *Nucleic Acid Res.* 20, 1193; and Debart *et al.* 1995, *Tetrahedron Lett.* 31, 3537. Alpha nucleosides (**1**) were 5'-protected for example by 5'-*O*-dimethoxytritylating **1** according to the standard procedure (see Oligonucleotide Synthesis: A Practical Approach, M.J. Gait Ed.; IRL Press, Oxford, 1984, p 27, and is incorporated by reference herin in its entirety) to yield **2** in high yield in the form of yellowish foams after silica gel column chromatography. To the stirred solution of the protected nucleoside **2** in 50 mL of dry THF and pyridine (4 eq), $AgNO_3$ (2.4 eq) was added. After 10 minutes tert-butyldimethylsilyl chloride (1.5 eq) was added and the reaction mixture was stirred at room temperature for 12 hours. The resulted suspension was filtered into 100 mL of 5% aq $NaHCO_3$. The solution was extracted with dichloromethane (2x100 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 and evaporated. The

residue containing **3** was purified by flash chromatography on silica gel. Compound **3** was then phosphitylated in the following way: To the ice-cooled stirred solution of protected nucleoside **3** (1 mmol) in dry dichloromethane (20 mL) under argon blanket was added dropwise via syringe the premixed solution of N,N-diisopropylethylamine (2.5eq) and 2-cyanoethyl N',N-diisopropylchlorophosphoramidite (1.2 eq) in dichloromethane (3 mL). Simultaneously via another syringe N-methylimidazole (1 eq) was added and stirring was continued for 2 hours at room temperature. After that the reaction mixture was again ice-cooled and quenched with 15 ml of dry methanol. After 5 min stirring, the mixture was concentrated in vacuo (<40°C) and purified by flash chromatography on silica gel to give corresponding phosphoroamidite **4**.

Alpha nucleoside phosphoramidites are incorporated into ribozymes using solid phase synthesis as described by Wincott *et al.*, 1995 *supra*, and is incorporated by reference herin in its entirety. The ribozymes are deprotected using the standard protocol described above.

Example 21: General procedure for the synthesis of 1-(β-D-erythrofuranosyl) nucleoside Phosphoramidites

Referring to Figure 37, 1-(β-D-erythrofuranosyl) nucleosides (**1**) are synthesized essentially as described by Szekeres *et al.*, 1977, *J. Carbohydr. Nucleosides Nucleotides.* 4, 147. 1-(β-D-erythrofuranosyl) nucleosides (**1**) were treated with $AgNO_3$ (2.4 eq). After 10 minutes tert-butyldimethylsilyl chloride (1.5 eq) was added and the reaction mixture was stirred at room temperature for 12 hours. The resulted suspension was filtered into 100 mL of 5% aq $NaHCO_3$. The solution was extracted with dichloromethane (2x100 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 and evaporated. The residue containing **2** was purified by flash chromatography on silica gel. Compound **2** was then phosphitylated in the following way: To the ice-cooled stirred solution of protected nucleoside **2** (1 mmol) in dry dichloromethane (20 mL) under argon blanket was added dropwise via syringe the premixed solution of N,N-diisopropylethylamine (2.5eq) and 2-cyanoethyl N',N-diisopropylchlorophosphoramidite (1.2 eq) in dichloromethane (3 mL). Simultaneously via another syringe N-methylimidazole (1 eq) was added and stirring was continued for 2 hours at room temperature. After that the reaction

mixture was again ice-cooled and quenched with 15 ml of dry methanol. After 5 min stirring, the mixture was concentrated in vacuo (<40°C) and purified by flash chromatography on silica gel to give corresponding phosphoroamidite **3**.

1-(β-D-erythrofuranosyl) nucleoside phosphoramidites are incorporated into ribozymes using solid phase synthesis as described by Wincott *et al.*, 1995 *supra*. The ribozymes aree deprotected using the standard protocol described above.

Example 22: General procedure for the synthesis of Inverted deoxyabasic 5'-O-Succinate and 5'-O-Phosphoramidite

Referring to to figure 38, commercially available 2-deoxyribose is converted to compound **1** in a two step process. In the first step, 2-deoxyribose is treated with a mixture of acetyl chloride and methanol. In the second step, the reaction mixture is treated with p-toluoyl chloride/pyridine mixture to yield **1**. Compund **1** is incubated with a mixture of triethyl silane and boron trifluoride in ethanol to yield compound **2**. Treatment of **4** with sodium methylate in methanol yield compound **3**. Reacting **3** with t-butyl-diphenyl-silyl chloride in pyridine yields compound **4**. The 3'-end of **4** is tritylated using 4,4'-dimethoxytrityl chloride in pyridine to yield compound **5**. The 5'-protecting group in **5** can be removed using a mixture of triethylamine/hydrogen fluoride/DCM to yield **6**.

A succinate group can be attached to the 5'-end of compound **6** by reacting the compound with a mixture of succinic anhydride and 4-dimethylaminopyridine to yield compound **7**.

Compound **6**, can be converted into a phosphoramidite by standard phosphitylation reaction described *supra* to yield compound **8**. Reaction of **8** with a standard phosphoramidite will yield a 5'-5'-inverted abasic deoxyribose linkage as shown in Figures 28C.

Example 23: General procedure for the synthesis of 3'-2'-inverted nucleotide or 3'-2'-inverted abasic linkage

Refering to Figure 34, a commercially available 5'-dimethoxytrityl-3'-silyl-containing nucleoside (**1**) is treated with a standard phosphitylation reagent

such as 2-cyanoethyl N',N- diisopropylchlorophosphoramidite to yield compound **2**. Reaction of compound **3**, wherein B is a natural or a modified base (described in Seliger *et al.*, Canadian Patent Application Publication No. 2,106,819., and is incorporated by reference herein), with compound **2** will result in a 3'-2'-inverted nucleotide linkage as shown in Figure 32B (3'-2'-inverted nucleotide).

Reaction of compound **3**, wherein B is H (see Figure 38; compound **7**), with compound **2** will result in a 3'-2'-inverted abasic linkage as shown in Figure 32B (3'-2'-inverted abasic).

Refering to Figure 38, compound **7** can be reacted with compound **2** in Figure 34 to yield a 3'-2'-inverted abasic deoxyribose linkage as shown in Figure 32B.

Alternatively, **7** (Figure 38) can be reacted with a standard nucleoside phosphoramidite to yield a 3'-3'-inverted abasic deoxyribose linkage as shown in Figure 32B.

Example 24: *In vitro* RNA Cleavage Activity of Ribozymes with 5'-Terminal Phosphorodithioate Modifications

Radio-labeling of Ribozymes and Substrates. Substrates were 5'-end-labeled using T4 Polynucleotide Kinase and γ-^{32}P-ATP.

Ribozyme Activity Assay. Ribozymes and 5'-^{32}P-end-labeled substrate were heated separately in reaction buffer (50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$) to 95 °C for 2 min, quenched on ice, and equilibrated to the final reaction temperature (37 °C) prior to starting the reactions. Reactions were carried out in enzyme excess, and were started by mixing ~1 nM substrate with the indicated amounts of ribozyme (50 nM-1 µM) to a final volume of 50 µL. Aliquots of 5 µL were removed at 1, 5, 15, 30, 60 and 120 min, quenched in formamide loading buffer, and loaded onto 15% polyacrylamide/8 M Urea gels. The fraction of substrate and product present at each time point was determined by quantitation of scanned images from a Molecular Dynamics PhosphorImager. Ribozyme cleavage rates were calculated from plots of the fraction of substrate remaining *vs* time using a double exponential curve fit

(Kaleidagraph, Synergy Software). The fast portion of the curve was generally 60-90% of the total reaction, so that observed cleavage rates (k_{obs}) were taken from fits of the first exponential.

Referring to Figure 39, ribozymes with either one or two phosphorodithioate substitutions were capable of catalyzing efficient RNA clevage reactions. The results show that modification of ribozymes at the 5'-end do not significantly effect the activity of ribozymes.

Uses

The 5'- and/or 3'-substituted enzymatic nucleic acids of this invention can be used to form stable molecules with enhanced activity as discussed above for use in enzymatic cleavage of target RNA. Such nucleic acids can be formed enzymatically using triphosphate forms by standard procedure. Administration of such nucleic acids into cells is by standard methods. Their *in vitro* utility is as known in the art. See Sullivan et al., PCT WO 94/ 02595.

Diagnostic uses

Enzymatic nucleic acids of this invention may be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of target RNA in a cell. The close relationship between ribozyme activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple ribozymes described in this invention, one may map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules). Other *in vitro* uses of ribozymes of this invention are well known

in the art, and include detection of the presence of mRNAs associated with disease condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a ribozyme using standard methodology.

In a specific example, ribozymes which can cleave only wild-type or mutant forms of the target RNA are used for the assay. The first ribozyme is used to identify wild-type RNA present in the sample and the second ribozyme will be used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA will be cleaved by both ribozymes to demonstrate the relative ribozyme efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates will also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis will require two ribozymes, two substrates and one unknown sample which will be combined into six reactions. The presence of cleavage products will be determined using an RNAse protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios will be correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

Other embodiments are within the following claims.

Table I

Characteristics of Ribozymes

Group I Introns
Size: ~150 to >1000 nucleotides.
Requires a U in the target sequence immediately 5' of the cleavage site.
Binds 4-6 nucleotides at 5' side of cleavage site.
Over 75 known members of this class. Found in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.

RNase P RNA (M1 RNA)
Size: ~290 to 400 nucleotides.
RNA portion of a ribonucleoprotein enzyme. Cleaves tRNA precursors to form mature tRNA.
Roughly 10 known members of this group all are bacterial in origin.

Hammerhead (HH) Ribozyme
Size: ~13 to 40 nucleotides.
Requires the target sequence UH immediately 5' of the cleavage site.
Binds a variable number of nucleotides on both sides of the cleavage site.
14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent (Figure 1 and 2).

Hairpin (HP) Ribozyme
Size: ~50 nucleotides.
Prefers the target sequence GUC immediately 3' of the cleavage site.
Binds 4-6 nucleotides at 5' side of the cleavage site and a variable number to the 3' side of the cleavage site.
Only 3 known member of this class. Found in three plant pathogen (satellite RNAs of the tobacco ringspot virus, arabis mosaic virus and chicory yellow mottle virus) which uses RNA as the infectious agent (Figure 3).

Hepatitis Delta Virus (HDV) Ribozyme
Size: 50 - 60 nucleotides (at present).
Sequence requirements not fully determined.
Binding sites and structural requirements not fully determined, although no sequences 5' of cleavage site are required.
Only 1 known member of this class. Found in human HDV (Figure 4).

***Neurospora* VS RNA (VS) Ribozyme**
Size: ~144 nucleotides (at present)
Cleavage of target RNAs recently demonstrated.
Sequence requirements not fully determined.
Binding sites and structural requirements not fully determined. Only 1 known member of this class. Found in *Neurospora* VS RNA (Figure 5).

Table II: RNA Synthesis Cycle (2.5 μmol Scale)

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidites	6.5	163 μL	2.5
S-Ethyl Tetrazole	23.8	238 μL	2.5
Acetic Anhydride	100	233 μL	5 sec
N-Methyl Imidazole	186	233 μL	5 sec
TCA	83.2	1.73 mL	21 sec
Iodine	8.0	1.18 mL	45 sec
Acetonitrile	NA	6.67 mL	NA

* Wait time does not include contact time during delivery.

Table III: Hammerhead Ribozyme Modifications at the 5'-Terminus or Positions 2.1-2.7

	Chemical Composition		
Ribozyme	**5'-Terminus**	**Positions 2.1-2.7**	**U4/U7**
Rz 1	OH	2'-ribose	2'-NH_2
Rz 2	OH	2'-deoxyribose	2'-NH_2
Rz 3	OH	2'-*O*-Me	2'-NH_2
Rz 4	OH	2'-*O*-Me, (2.3-2.7 P=S)	2'-NH_2
Rz 5 (inactive)*	OH	2'-*O*-Me, (2.3-2.7 P=S)	2'-NH_2
Rz 6	NH_2	2'-*O*-Me	2'-NH_2
Rz 7 (inactive)	NH_2	2'-*O*-Me	2'-NH_2
Rz 8	NH_2	2'-*O*-Me	2'-*C*-allyl/*O*-Me
Rz 9 (inactive)	NH_2	2'-*O*-Me	2'-*C*-allyl/*O*-Me
Rz 10	OH	2'-*O*-Me, (2.3-2.7 P=S)	2'-*C*-allyl/*O*-Me
Rz 11 (inactive)	OH	2'-*O*-Me, (2.3-2.7 P=S)	2'-*C*-allyl/*O*-Me

* Catalytically inactive ribozyme cores were produced by substituting 2'-*O*-Me U at positions G5 and A 14.

Table IV. Cleavage rate of the substrate 3 by the ribozymes 6, 7 and RPI.2972.

Ribozyme	6	7	RPI 2972[b]
$k_{obs}.(min^{-1})^{a}$	0.013	0.145	0.023

[a] [Rz] = 500 nM, [3] ~ 1 nM, 50 nM tris.HCl pH 8.0, 25°C, 40 nM Mg^{++}.

[b] gsusususuc cc*U* Gau Gag gcc gaa agg ccG aaA uuc ucc iB

Table V: Comparative Catalytic Activities for U4/U7-amino-Containing-Hammerhead Ribozymes

Ribozyme	k_{obs} (min^{-1}) [Rz] = 40nM	k_{obs} (min^{-1}) [Rz] = 500nM*
Rz 1	0.128±0.032	0.140±0.015
Rz 2	0.019±0.002	0.023±0.002
Rz 3	0.163±0.012	0.200±0.015
Rz 4	0.108±0.001	0.150±0.003
Rz 6	0.131±0.007	0.149±0.007

*Neither U4-*C*-allyl containing ribozymes nor ribozymes containing inactivating nucleotide changes exhibited measurable activity under the standard conditions employed for these measurements. k_{obs} is derived from two independent assays and is expressed as average ± range. Values in parentheses express the cleavage rate as a percentage of the control cleavage rate using **Rz 3** at equivalent concentrations.

Table VI: 5'-Amino-5'-deoxynucleotide incorporation

Experiment	Coupling of 5	Coupling of 2'-*O*-Me-G	Desilylating Reagent	Crude AU	% FLP
4298	600 s	600s	HF/TEA	355.3	14.8
4298	600 s	600s	TBAF	387.3	22.2
4523	600 s	900 s	TBAF	401.7	21.4
4545	600 s	450 s	TBAF	447.8	23.8
4649	300 s	300 s	TBAF	455.4	27.3

Table VII. Inhibition of Rat Smooth Cell Proliferation in Culture

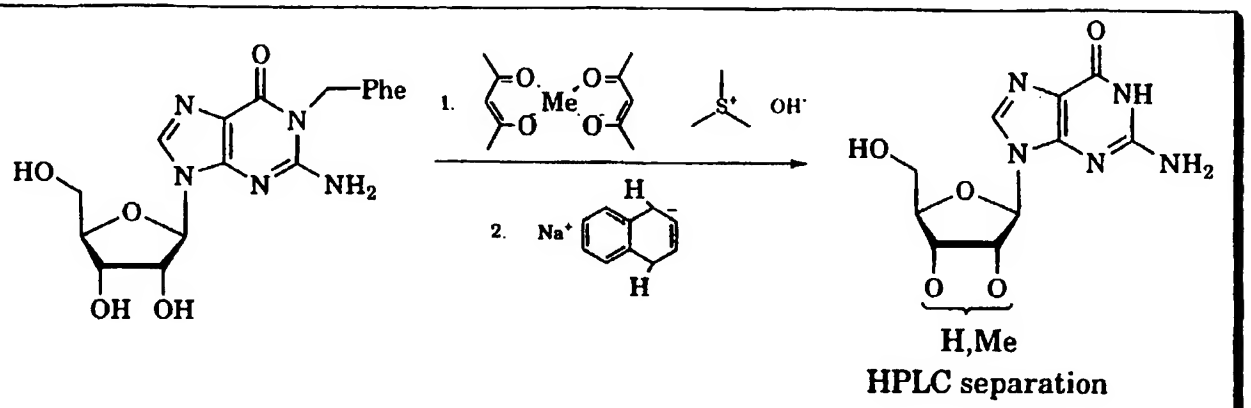
Relative Proliferation Index

[Ribozyme] nM

Ribozyme		50 nM	100 nM	200 nM
Rz 1		83 ± 9	75 ± 12	57 ± 10
Rz 2		104 ± 5	80 ± 6	58 ± 7
Rz 3		103 ± 7	82 ± 13	57 ± 10
Rz 4		82 ± 11	31 ± 11	24 ± 5
Rz 5		83 ± 5	31 ± 8	38 ± 5
Rz 6		88 ± 7	24 ± 7	18 ± 6
Rz 7		104 ± 3	69 ± 13	40 ± 6
Rz 8		106 ± 3	71 ± 9	47 ± 7
Rz 9		103 ± 6	87 ± 7	56 ± 7
Rz 10		79 ± 9	17 ± 5	26 ± 12
Rz 11		93 ± 12	69 ± 11	32 ± 9

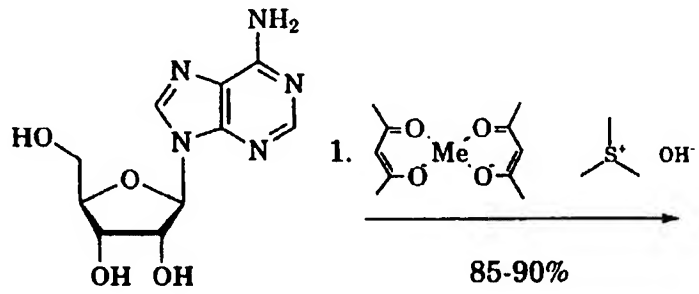
Values given represent the percentage of proliferating cell nuclei relative to stimulated lipid-treated cell controls. Mean values of at least 9 experimental points were used to obtain the relative proliferative index for each treatment protocol. Numbers in parentheses represent the standard deviation of the mean values. Unstimulated control values were 5 (± 2)%. The percentage of proliferating nuclei in the serum stimulated control wells was 72 (± 6)%.

Table VIII : Methylation of N^1-Benzyl Guanosine with acethylacetonates



Metal	2'-O-Me-G^{bzl}	:	3'- O-Me-G^{bzl}	2'-O-Me-G yield
Cu	40	:	42	32
Mg	65	:	7	45
Ag	70	:	8	70

Table IX: Metal - Directed methylation of Adenosine



Metal	*2'-O-Me-A*	:	*3'-O-Me-A*	*2'-O-Me -A yield*
Fe	1	:	1	45%
Cu	2	:	1	60%
Ag	4	:	1	72%
Sr	8	:	1	80%

CLAIMS

1. A process for the synthesis of a 2'-*O*-methyl nucleoside, comprising the step of contacting a 2,2'-anhydro-1-(β-D-arabinofuranosyl) base with methanol and a Lewis acid under conditions suitable for formation of said nucleoside.

2. A process for the synthesis of 3'-*O*-methyl nucleoside, comprising the step of contacting 2,3'-anhydro-1-(β-D-arabinofuranosyl) base with methanol and a Lewis acid under conditions suitable for formation of said nucleoside.

3. A process for the synthesis of 5'-*O*-methyl nucleoside, comprising the step of contacting 2,5'-anhydro-1-(β-D-arabinofuranosyl) base with methanol and a Lewis acid under conditions suitable for formation of said nucleoside.

4. The process according to any of claims 1-3, wherein said Lewis acid is trimethylborate.

5. The process according to any of claims 1-3, wherein said Lewis acid is boron trifluoride.

6. A process for the synthesis of 2'-*O*-methyl cytidine nucleoside, comprising the steps of:

a) contacting 2,2'-anhydro-1-(β-D-arabinofuranosyl) cytosine with methanol and trimethylborate to form a mixture;

b) heating said mixture at a temperature and for a time sufficient to break the 2,2'-linkage within said 2,2'-anhydro-1-(β-D-arabinofuranosyl) cytosine;

c) cooling and drying to form a dried mixture;

d) contacting said dried mixture with N,N-dimethylformamide and acetic anhydride to form a crude mixture containing said 2'-*O*-methyl cytidine nucleoside; and

e) purifying said nucleoside from said crude mixture.

7. A process for the synthesis of 2'-*O*-methyl cytidine nucleoside, comprising the steps of:

a) contacting 2,2'-anhydro-1-(β-D-arabinofuranosyl) cytosine with methanol, boron trifluoride-methanol and trimethylborate to form a mixture;

b) heating said mixture at a temperature and for a time sufficient to break the 2,2'-linkage within said 2,2'-anhydro-1-(β-D-arabinofuranosyl) cytosine;

c) cooling and drying to form a dried mixture;

d) contacting said dried mixture with N,N-dimethylformamide and acetic anhydride to form a crude mixture containing said 2'-*O*-methyl cytidine nucleoside; and

e) purifying said nucleoside from said crude mixture.

8. A process for the synthesis of 2'-*O*-methyl uridine nucleoside, comprising the steps of:

a) contacting 2,2'-anhydro-1-(β-D-arabinofuranosyl) uracil with methanol, trimethylborate and boron trifluoride-methanol to form a mixture;

b) heating said mixture at a temperature and for a time sufficient to break the 2,2'-linkage within said 2,2'-anhydro-1-(β-D-arabinofuranosyl) uracil;

c) cooling and drying to form a crude mixture containing said 2'-*O*-methyl uridine nucleoside; and

d) purifying said nucleoside from said crude mixture.

9. A process for the synthesis of 2'-*O*-methyl uridine nucleoside, comprising the steps of:

a) contacting 2,2'-anhydro-1-(β-D-arabinofuranosyl)uracil with methanol and trimethylborate to form a mixture;

b) heating said mixture at a temperature and for a time sufficient to break the 2,2'-linkage within said 2,2'-anhydro-1-(β-D-arabinofuranosyl) uracil;

c) cooling and drying to form a crude mixture containing said 2'-*O*-methyl uridine nucleoside; and

d) purifying said nucleoside from said crude mixture.

10. The process according to any of claims 8 or 9, wherein said heating was at between 80°C to 135°C for 5 to 24 hours.

11. A process for the synthesis of 3'-*O*-methyl pyrimidine nucleoside, comprising the steps of:

a) contacting 2,3'-anhydro-1-(β-D-arabinofuranosyl) pyrimidine with methanol and trimethylborate to form a mixture;

b) heating said mixture at a temperature and for a time sufficient to break the 2,3'-linkage within said 2,3'-anhydro-1-(β-D-arabinofuranosyl) pyrimidine;

c) cooling and drying to form a crude mixture containing said 3'-*O*-methyl pyrimidine nucleoside; and

d) purifying said nucleoside from said crude mixture.

12. A process for the synthesis of 5'-*O*-methyl pyrimidine nucleoside, comprising the steps of:

a) contacting 2,5'-anhydro-1-(β-D-arabinofuranosyl)pyrimidine with methanol and trimethylborate;

b) heating said at a temperature and for a time sufficient to break the 2,5'-linkage within said 2,5'-anhydro-1-(β-D-arabinofuranosyl) pyrimidine;

c) cooling and drying to form a crude mixture containing said 5'-*O*-methyl pyrimidine nucleoside; and

d) purifying said nucleoside from said crude mixture.

13. The process according to any of claims 6, 7, 11, or 12, wherein said heating was at between 80°C to 135°C for 15 to 48 hours.

14. A process for the synthesis of a 2'-*O*-methyl adenosine nucleoside, comprising the step of contacting a solution of N^4-acetyl-5',3'-di-*O*-acetyl-2'-*O*-methyl cytidine with a Lewis acid under conditions suitable for the formation of said nucleoside.

15. A process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of:

a) methylating 2-amino-6-chloropurine riboside by contacting said 2-amino-6-chloropurine riboside with sodium hydride, dimethylformamide and methyl iodide under conditions suitable for the formation of 2'-*O*-methyl-2-amino-6-chloropurine riboside;

b) contacting said 2'-*O*-methyl-2-amino-6-chloropurine riboside with 1,4-diazabicyclo(2.2.2) octane and water under conditions suitable for the formation of said 2'-*O*-methyl guanosine nucleoside in a crude form; and

c) purifying said 2'-*O*-methyl guanosine nucleoside from said crude form.

16. A process for the synthesis of 2'-*O*-methyl adenosine nucleoside, comprising the steps of:

a) methylating 2-amino-6-chloropurine riboside by contacting said 2-amino-6-chloropurine riboside with sodium hydride, dimethylformamide and methyl iodide under conditions suitable for the formation of 2'-*O*-methyl-2-amino-6-chloropurine riboside;

b) contacting said 2'-*O*-methyl-2-amino-6-chloropurine riboside with acetic anhydride, 4-dimethylaminopyridine and triethylamine under conditions suitable for the formation of 3',5'-di-*O*-acetyl-2'-*O*-methyl-6-chloro-2-aminopurine riboside;

c) deaminating said 3',5'-di-*O*-acetyl-2'-*O*-methyl-6-chloro-2-aminopurine riboside with isoamyl nitrite and tetrahydrofuran to form 3',5'-di-*O*-acetyl-2'-*O*-methyl-6-chloropurine;

d) aminating said 3',5'-di-*O*-acetyl-2'-*O*-methyl-6-chloropurine with ammonia to form 2'-*O*-methyl adenosine nucleoside in a crude form; and

e) purifying said 2'-*O*-methyl adenosine nucleoside from said crude form.

17. A process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of:

a) contacting 2,6-diaminopurine nucleoside with anhydrous pyridine and tetraisopropyl D-silyl chloride under conditions suitable for the formation of 2,6-diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine;

b) methylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine with anhydrous DMF and methyl iodide under conditions suitable for the formation of 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-2'-*O*-methyl-β-D-ribofuranosyl) purine;

c) acylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine with anhydrous pyridine and isobutyryl chloride under conditions

suitable for the formation of *2,6-Diamino-N²-isobutyryl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-O-methyl-β-D-ribofuranosyl) purine*;

d) deaminating and desilylating said *2,6-Diamino-N²-isobutyryl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine* under conditions suitable for the formation of N^2-isobutyryl-2'-*O*-methyl guanosine nucleoside in a crude form;

e) purifying said N^2-isobutyryl-2'-*O*-methyl guanosine nucleoside from said crude form; and

f) deblocking said N^2-isobutyryl-2'-*O*-methyl guanosine nucleoside under suitable conditions to form said 2'-*O*-methyl guanosine nucleoside.

18. A process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of:

a) contacting 2,6-diaminopurine nucleoside with anhydrous pyridine and tetraisopropyl D-silyl chloride under conditions suitable for the formation of 2,6-Diamino-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine;

b) methylating said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine with anhydrous DMF and methyl iodide under conditions suitable for the formation of 2,6-Diamino-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-*O*-methyl-β-D-ribofuranosyl) purine;

c) acylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine with anhydrous pyridine and isopropylphenoxyacetyl chloride under conditions suitable for the formation of *2,6-Diamino-N²-*

isopropylphenoxyacetyl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine;

d) deaminating and desilylating said *2,6-Diamino-N--isopropylphenoxyacetyl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine* under conditions suitable for the formation of N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside in a crude form;

e) purifying said N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside from said crude form; and

f) deblocking said N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside under suitable conditions to form said 2'-*O*-methyl guanosine nucleoside.

19. A process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of:

a) contacting guanosine with N,N-dimethylformamide dibenzyl acetal under conditions suitable for the formation of N1-benzyl guanosine;

b) methylating said N1-benzyl guanosine by contacting said N1-benzyl guanosine with silver acetylacetonate, trimethylsulphonium hydroxide and dimethylformamide under conditions suitable for the formation of N1-benzyl-2'-*O*-methyl guanosine in a crude form;

c) purifying said N1-benzyl-2'-*O*-methyl guanosine from said crude form;

d) removing the N1-benzyl protection from said N1-benzyl-2'-*O*-methyl guanosine by contacting said N1-benzyl-2'-*O*-methyl guanosine with sodium naphthalene under conditions suitable for the formation of 2'-*O*-methyl guanosine nucleoside in a crude form; and

e) purifying said 2'-*O*-methyl guanosine from said crude form.

20. A process for the synthesis of 2'-*O*-methyl adenosine nucleoside, comprising the steps of:

a) methylating adenosine by contacting said adenosine with dimethylformamide, silver acetylacetonate and trimethylsulphonium hydroxide under conditions suitable for the formation of 2'-*O*-methyl adenosine in a crude form; and

b) purifying said 2'-*O*-methyl adenosine from said crude form.

21. A process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of:

a) contacting guanosine with N,N-dimethylformamide dibenzyl acetal under conditions suitable for the formation of N1-benzyl guanosine;

b) methylating said N1-benzyl guanosine by contacting said N1-benzyl guanosine with magnesium acetylacetonate, trimethylsulphonium hydroxide and dimethylformamide under conditions suitable for the formation of N1-benzyl-2'-*O*-methyl guanosine in a crude form;

c) purifying said N1-benzyl-2'-*O*-methyl guanosine from said crude form;

d) removing the N1-benzyl protection from said N1-benzyl-2'-*O*-methyl guanosine by contacting said N1-benzyl-2'-*O*-methyl guanosine with sodium naphthalene under conditions suitable for the formation of 2'-*O*-methyl guanosine nucleoside in a crude form; and

e) purifying said 2'-*O*-methyl guanosine nucleoside from said crude form.

22. A process for the synthesis of 2'-*O*-methyl adenosine nucleoside, comprising the steps of:

a) methylating adenosine by contacting said adenosine with dimethylformamide, magnesium acetylacetonate and trimethylsulphonium hydroxide under conditions suitable for the formation of 2'-*O*-methyl adenosine in a crude form; and

b) purifying said 2'-*O*-methyl adenosine from said crude form.

23. A process for the synthesis of 2'-*O*-methyl adenosine nucleoside, comprising the steps of:

a) methylating adenosine by contacting said adenosine with dimethylformamide, strontium acetylacetonate and trimethylsulphonium hydroxide under conditions suitable for the formation of 2'-*O*-methyl adenosine in a crude form; and

b) purifying said 2'-*O*-methyl adenosine from said crude form.

24. A process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of:

a) contacting 2,6-diaminopurine nucleoside with anhydrous pyridine and TIPSCl under conditions suitable for the formation of 2,6-diamino-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine;

b) methylating said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine with anhydrous DMF and methyl iodide under conditions suitable for the formation of 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine;

c) acylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine with anhydrous pyridine and isobutyryl chloride under conditions

suitable for the formation of *2,6-Diamino-N^2-isobutyryl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-methyl-β-D-ribofuranosyl) purine*;

d) deaminating and desilylating said *2,6-Diamino-N^2-isobutyryl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-O-methyl-β-D-ribofuranosyl) purine* under conditions suitable for the formation of N2-isobutyryl-2'-*O*-methyl guanosine nucleoside in a crude form; and

e) purifying said N2-isobutyryl-2'-*O*-methyl guanosine nucleoside from said crude form.

25. A process for the synthesis of 2'-*O*-methyl guanosine nucleoside, comprising the steps of:

a) contacting 2,6-diaminopurine nucleoside with anhydrous pyridine and TIPSCl under conditions suitable for the formation of 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine;

b) methylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-*O*-tetraisopropyldisiloxane-(1,3-diyl)-β-D-ribofuranosyl) purine with anhydrous DMF and methyl iodide under conditions suitable for the formation of 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine;

c) acylating said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-*O*-methyl-β-D-ribofuranosyl) purine by contacting said 2,6-Diamino-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-*O*-methyl-β-D-ribofuranosyl) purine with anhydrous pyridine and isopropylphenoxyacetyl chloride under conditions suitable for the formation of *2,6-Diamino-N^2-isopropylphenoxyacetyl-9-(3',5'-O-tetraisopropyldisiloxane-(1,3-diyl)-2'-O-methyl-β-D-ribofuranosyl) purine*;

d) deaminating and desilylating said *2,6-Diamino-N^2-isopropylphenoxyacetyl-9-(3,5-O-tetraisopropyldisiloxane-(1,3-diyl)-2-O-*

methyl-β-D-ribofuranosyl) purine under conditions suitable for the formation of N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside in a crude form; and

e) purifying said N^2-*isopropylphenoxyacetyl*-2'-*O*-methyl guanosine nucleoside from said crude form.

26. A method for synthesis of an enzymatic nucleic acid, comprising the steps of:

providing a 3' and a 5' portion of said enzymatic nucleic acid having independent chemically reactive groups at the 5' and 3' positions, respectively, under conditions in which a covalent bond is formed between said 3' and 5' portions by said chemically reactive groups, said bond being selected from the group consisting of, disulfide, morpholino, amide, ether, thioether, amine, a double bond, sulfonamide, ester, carbonate, hydrazone, said bond not being a natural bond formed between a 5' phosphate group and a 3' hydroxyl group.

27. The method of claim 26, wherein said nucleic acid has a hammerhead motif and said 3' and 5' positions each have said chemically reactive groups in or immediately adjacent to the stem II region.

28. The method of claim 26, wherein one said chemically reactive group is $(CH_2)_nSH$ and the other chemically reactive group is $(CH_2)_nSH$, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

29. The method of claim 26, wherein one said chemically reactive group is $(CH_2)_nNH_2$ and the other chemically reactive group is ribose, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

30. The method of claim 26, wherein one said chemically reactive group is $(CH_2)nNH_2$ and the other chemically reactive group is COOH, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

31. The method of claim 26, wherein one said chemically reactive group is $(CH_2)nX$ and the other chemically reactive group is $(CH_2)_nOH$ or $(CH_2)_nSH$; wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different; X is halogen.

32. The method of claim 26, wherein one said chemically reactive group is $(CH_2)nNH_2$ and the other chemically reactive group is CHO, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

33. The method of claim 26, wherein one said chemically reactive group is $(CH_2)nPPh_3$ and the other chemically reactive group is CHO, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

34. The method of claim 26, wherein one said chemically reactive group is $(CH_2)nNH_2$ and the other chemically reactive group is $(CH_2)nSO_2Cl$, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

35. The method of claim 26, wherein one said chemically reactive group is $(CH_2)nOH$ and the other chemically reactive group is COOH, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

36. The method of claim 26, wherein one said chemically reactive group is $(CH_2)_nCOH$ and the other chemically reactive group is $(CH_2)_nNH_2$, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

37. The method of claim 26, wherein one said chemically reactive group is $(CH_2)_nCOX$ and the other chemically reactive group is $(CH_2)_nOH$, wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different.

38. The method of claim 29, wherein said conditions include provision of $NaIO_4$ in contact with said ribose, and subsequent provision of $NaBH_4$ or $NaCNBH_3$.

39. The method of claim 30, wherein said conditions include provision of a coupling reagent.

40. A mixture comprising 5' and 3' portions of an enzymatic nucleic acid having a 3' and 5' chemically reactive group respectively selected from the group consisting of $(CH_2)_nSH$, $(CH_2)_nNH_2$, ribose, COOH, $(CH_2)_nX$, $(CH_2)_nPPh_3$, CHO, $(CH_2)_nSO_2Cl$, $(CH2)_nCOX$, $(CH_2)_nX$, $(CH_2)_nOH$, $(CH2)_nCOH$, and $(CH_2)_nSH$; wherein each n independently is an integer from 0 to 10 inclusive and may be the same or different and X is halogen.

41. An enzymatic nucleic acid molecule comprising a 5'- and/or a 3'-cap structure, wherein said structure is not a 5'-5'-linked inverted nucleotide or a 3'-3'-linked inverted nucleotide.

42. The enzymatic nucleic acid molecule of claim 41, wherein said nucleic acid is in a hammerhead motif.

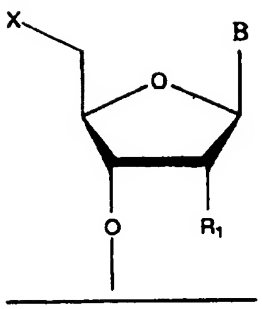
43. The enzymatic nucleic acid molecule of claim 41, wherein said nucleic acid is in a hairpin, hepatitis delta virus, group I intron, VS RNA or RNase P RNA motif.

44. The enzymatic nucleic acid molecule of claim 41, wherein said nucleic acid comprises a 5'-cap structure.

45. The enzymatic nucleic acid molecule of claim 41, wherein said nucleic acid comprises a 3'-cap structure.

46. The enzymatic nucleic acid molecule of claim 41, wherein said nucleic acid comprises a 5'-cap structure and a 3'-cap structure.

47. The enzymatic nucleic acid molecule of claim 41, wherein said 5'-cap structure has the formula:



wherein, X represents H, alkyl, amino alkyl, halo, Trihalomethyl [CX_3 (wherein X= F, Br, or Cl)], N_3, NH_2, NHR, NR_2 [wherein R=alkyl(C1-22), acyl (C1-22), substituted (wherein substituted=alkyl, alkoxy, amino, halogen or the like) or unsubstituted aryl], NO_2, $CONH_2$, COOR [wherein R=alkyl(C1-22), acyl (C1-22), substituted (wherein substituted=alkyl, alkoxy, amino, halogen or the like) or unsubstituted aryl], SH, SR [wherein R=alkyl(C1-22), acyl (C1-22), substituted (wherein substituted=alkyl, alkoxy, amino, halogen or the like) or unsubstituted aryl], OR [R=alkyl(C1-22), acyl (C1-22), substituted (wherein substituted=alkyl, alkoxy, amino, halogen or the like) or unsubstituted aryl], ONHR [wherein R=alkyl(C1-22), acyl (C1-22), substituted (wherein substituted=alkyl, alkoxy, amino, halogen or the like) or unsubstituted aryl], ONR_2 [wherein R=alkyl(C1-22), acyl (C1-22), substituted (wherein substituted=alkyl, alkoxy, amino, halogen or the like) or unsubstituted aryl], PO_4^{2-}, PO_3S^{2-}, $PO_2S_2^{2-}$, POS_3^{2-}, PO_3NH^{2-}, PO_3NHR^- [R=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl], NO_2, $CONH_2$, COOR [wherein R=alkyl (C1-22), acyl (C1-22), substituted (alkyl, amino, alkoxy, halogen, or the like) or unsubstituted aryl];

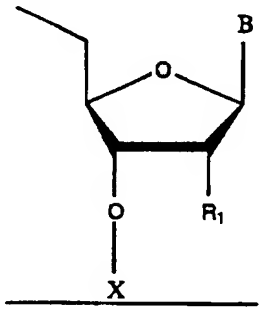
B represents a nucleotide base or H; and

R1 represents H, O-alkyl, C-alkyl, halo, NHR [wherein R=alkyl(C1-22), acyl (C1-22), substituted (wherein substituted=alkyl, alkoxy, amino, halogen or the like) or unsubstituted aryl], or OCH_2SCH_3 (methylthiomethyl).

48. The enzymatic nucleic acid molecule of claim 41, wherein said 5'-cap is selected from the group consisting of:

4',5'-methylene nucleotide; 1-(β-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 12-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; α-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moeity; 5'-5'-inverted abasic moeity; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate; phosphorothioate and/or phosphorodithioate; bridging and/or nonbridging methylphosphonate; and a 5'-mercapto moeity.

49. The enzymatic nucleic acid molecule of claim 41, wherein said 3'-cap structure has the formula:



wherein, X represents 4'-thio nucleoside, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotide, α-nucleotide, modified base nucleotide, phosphorodithioate linkage, *threo*-pentofuranosyl nucleotide, acyclic 3',4'-seco nucleotide, acyclic 3,4-dihydroxybutyl nucleotide, acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moeity, 3'-3'-inverted abasic moeity, 3'-2'-inverted nucleoside moeity, 3'-2'-inverted abasic moeity, 1,4-butanediol, 3'-

phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 3'-phosphorothioate, or bridging methylphosphonates moeity;

B represents a nucleotide base or H;

and R1 represents H, O-alkyl, *C*-alkyl, halo, NHR [wherein R=alkyl(C1-22), acyl (C1-22), substituted (wherein substituted=alkyl, alkoxy, amino, halogen or the like) or unsubstituted aryl], or OCH_2SCH_3 (methylthiomethyl).

50. The enzymatic nucleic acid molecule of claim 41, wherein said cap is selected from the group consisting of:

4'-thio nucleoside, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotide; α-nucleotide; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide; 3'-3'-inverted nucleotide moeity; 3'-3'-inverted abasic moeity; 3'-2'-inverted nucleotide moeity; 3'-2'-inverted abasic moeity; 1,4-butanediol; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; and a bridging methylphosphonates moeity.

51. The enzymatic nucleic acid molecule of any of claims 41 or 46, wherein said cap structures are the same or different.

52. The enzymatic nucleic acid molecule of any one of claims 41-5o, wherein said nucleic acid comprises at least one ribonucleotide residue.

53. The enzymatic nucleic acid of 42, wherein said nucleic acid comprises at least five ribose residues; at least three 5' terminal nucleotides are substituted with phosphorothioate linkages; position No. 4 of said nucleic acid is substituted with a 2'-*C*-allyl modification; and wherein said nucleic acid comprises at least ten 2'-*O*-methyl modifications.

54. The enzymatic nucleic acid of claim 42, wherein said nucleic acid comprises at least five ribose residues; said nucleic acid comprises a 2'-amino modification at position No. 4 and/or at position No. 7; and wherein said nucleic acid comprises at least ten 2'-*O*-methyl modifications.

55. The enzymatic nucleic acid of claim 42, wherein said nucleic acid comprises at least five ribose residues; said nucleic acid comprises abasic substitution at position No. 4 and/or at position No. 7; and wherein said nucleic acid comprises at least ten 2'-*O*-methyl modifications.

56. The enzymatic nucleic acid of claim 42, wherein said nucleic acid comprises at least five ribose residues; said nucleic acid comprises 6-methyl uridine substitutions at position No. 4 and/or at position No. 7; and wherein said nucleic acid comprises at least ten 2'-*O*-methyl modifications.

57. The enzymatic nucleic acid of claim 42, wherein said nucleic acid comprises at least five ribose residues; said nucleic acid comprises pyridin-4-one substitution at position No. 4 and/or at position No. 7; and wherein said nucleic acid comprises at least ten 2'-*O*-methyl modifications.

58. The enzymatic nucleic acid of claim 42, wherein said nucleic acid comprises a stem II region of length greater than or equal to two base-pairs.

59. The enzymatic nucleic acid of claim 43, wherein said hairpin nucleic acid comprises a stem IV region of length greater than or equal to two base-pairs.

60. The enzymatic nucleic acid of claim 43, wherein said hairpin nucleic acid comprises a stem II region of length between three and seven base-pairs.

61. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a 3'-3' linked inverted ribose.

62. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a 3'-2' linked inverted ribose.

63. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a 3'-2' linked inverted nucleotide.

64. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a 5'-5' linked inverted ribose.

65. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a 5'-amino group.

66. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a phosphoramidate group.

67. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a phosphorodithioate group.

68. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a phosphorothioate group.

69. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a 4'-thio group.

70. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a 1,3-diamino-2-propyl group.

71. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a L-nucleotide group.

72. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a alpha-nucleotide group.

73. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is an *threo*-pentafuranosyl group.

74. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a acyclic 3,4-dihydroxybutyl group.

75. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a acyclic 3,5-dihydroxypentyl group.

76. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 5'-cap is a 1-(β-D-erythrofuranosyl) nucleotide group.

77. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a 4'-thio group.

78. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a L-nucleotide group.

79. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a alpha-nucleotide group.

80. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a phosphorodithioate group.

81. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a phosphorothioate group.

82. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a acyclic 3,4-dihydroxybutyl group.

83. The enzymatic nucleic acid of any one of claims 41-50 and 53-60, wherein said 3'-cap is a acyclic 3,5-dihydroxypentyl group.

84. The enzymatic nucleic acid of claim 41, 42 or 46, wherein said 5'-cap is a 5'-amino group and said 3'-cap is a 3'-3'-inverted abasic ribose group.

85. The enzymatic nucleic acid of claim 41, 42 or 46, wherein said 5'-cap is a 5'-phosphorodithioate group and said 3'-cap is a 3'-3'-inverted abasic ribose group.

86. The enzymatic nucleic acid of claim 41, 42 or 46, wherein said 5'-cap is a 5'-amino group and said 3'-cap is a 3'-2'-inverted abasic ribose group.

87. The enzymatic nucleic acid of claim 41, 42 or 46, wherein said 5'-cap is a 5'-phosphorodithioate group and said 3'-cap is a 3'-2'-inverted abasic ribose group.

88. A mammalian cell comprising a compound of any one of claims 41-50 and 53-60.

89. The cell of claim 88, wherein said cell is a human cell.

90. Method for producing an enzymatic nucleic acid molecule having activity to cleave an RNA or single-stranded DNA molecule, comprising the step of forming said enzymatic molecule with a 5'- and/or a 3'-cap structure, wherein said structure is not a 5'-5'-linked inverted nucleotide or a 3'-3'-linked inverted nucleotide.

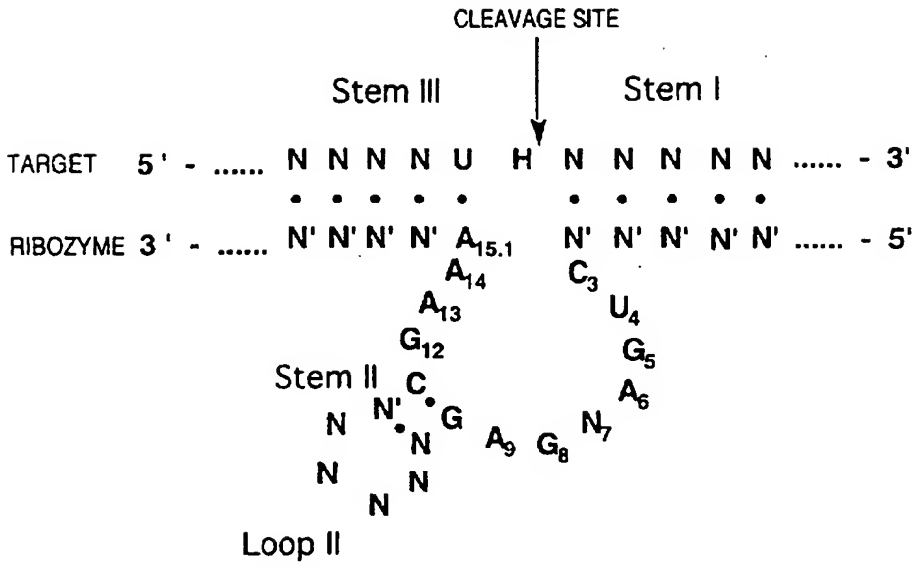
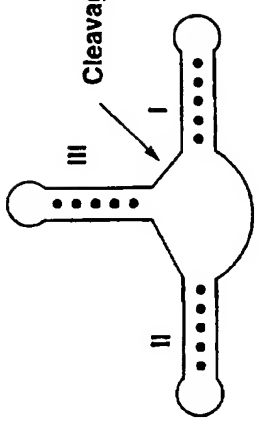

CLEAVAGE SITE
Stem III
Stem I
TARGET 5' - ..... N N N N U H N N N N N ..... - 3'
RIBOZYME 3' - ..... N' N' N' N' A15.1 N' N' N' N' N' ..... - 5'
A14
C3
A13
U4
G12
G5
Stem II
C
A6
N'
G
N7
A9
G8
Loop II

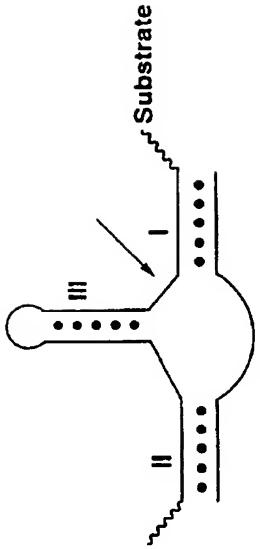
FIG. 1.

FIG. 2A



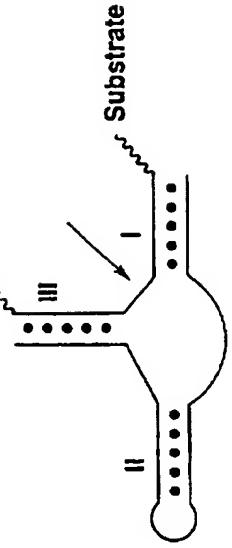
a

FIG. 2B



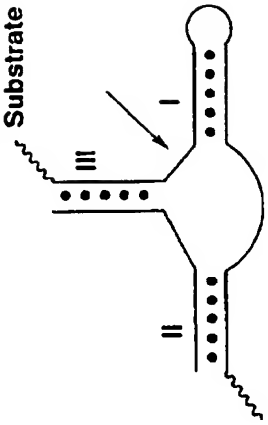
b

FIG. 2C



c

FIG. 2D



d

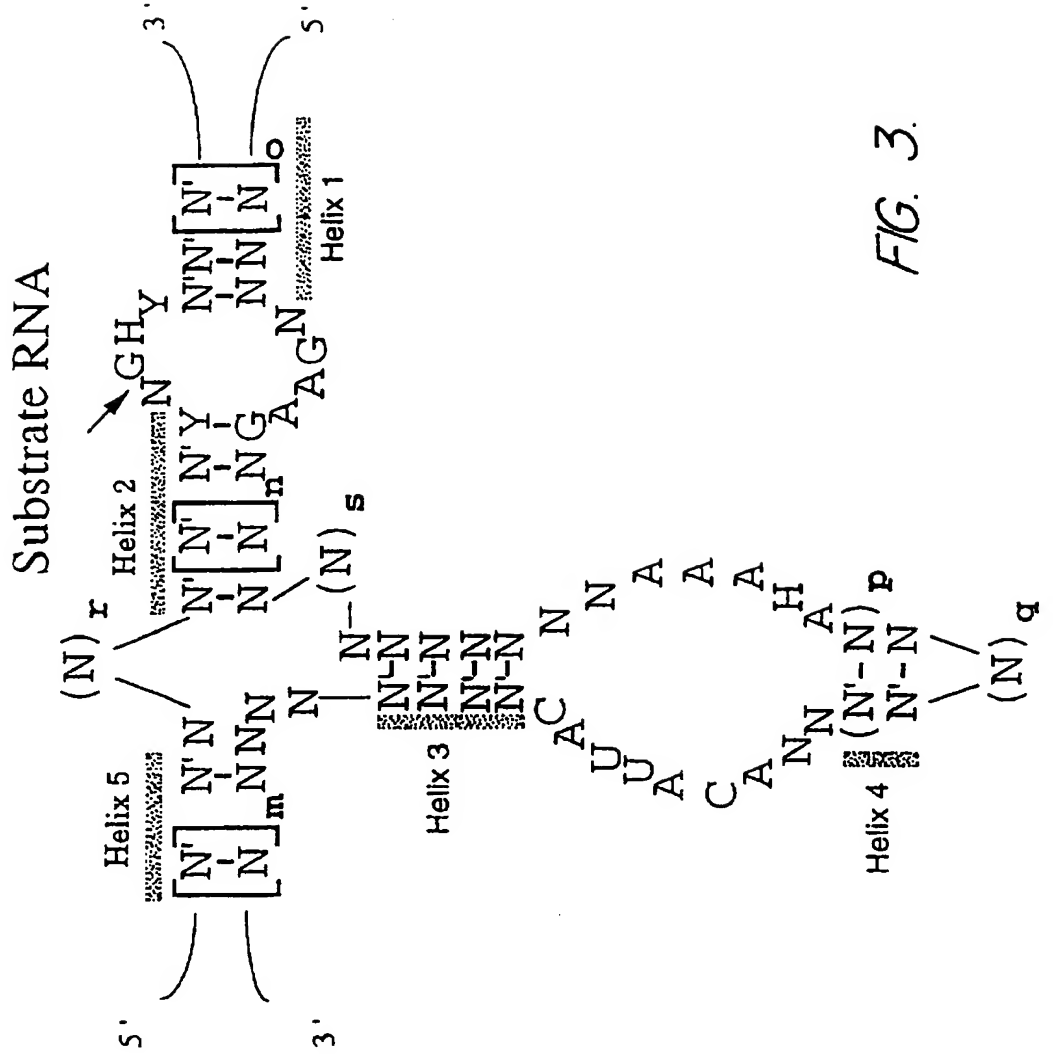


FIG. 3.

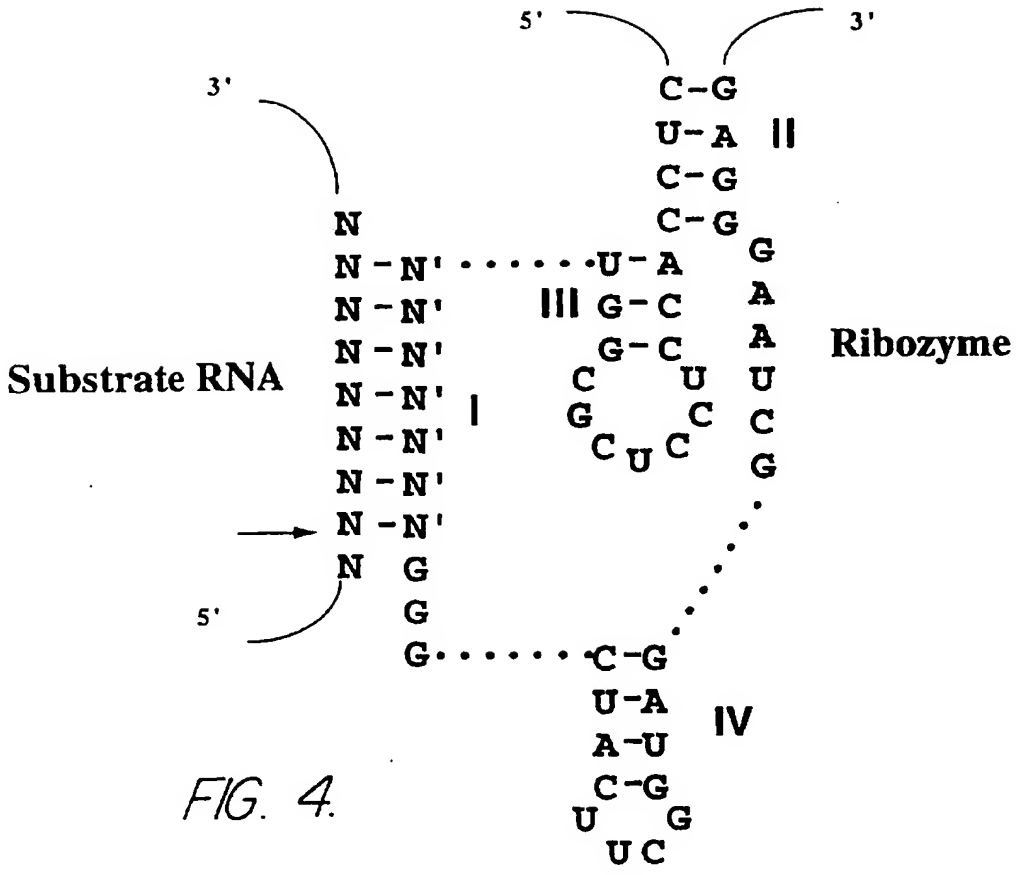


FIG. 4.

NEUROSPORA VS RNA ENZYME

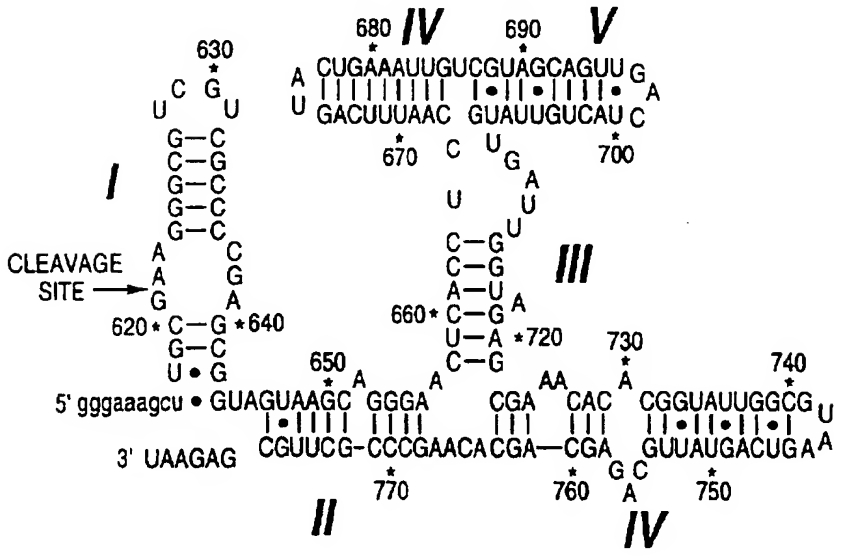
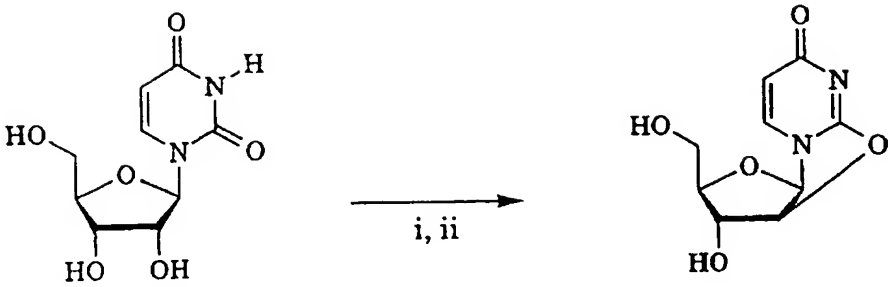


FIG. 5.

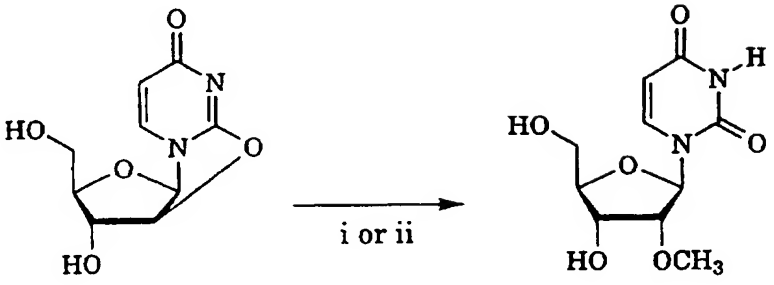
FIG. 6



1 2

i. diphenylcarbonate, $NaHCO_3$ / DMF 110°C
ii. MeOH reflux

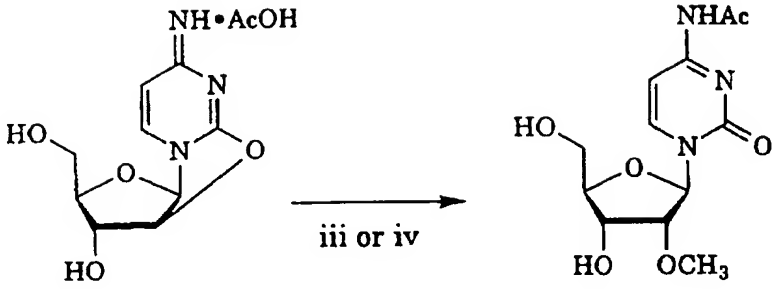
FIG. 7



2 3

i. $B(OCH_3)_3$, BF_3•MeOH / MeOH
ii. $B(OCH_3)_3$ / MeOH

FIG. 8.

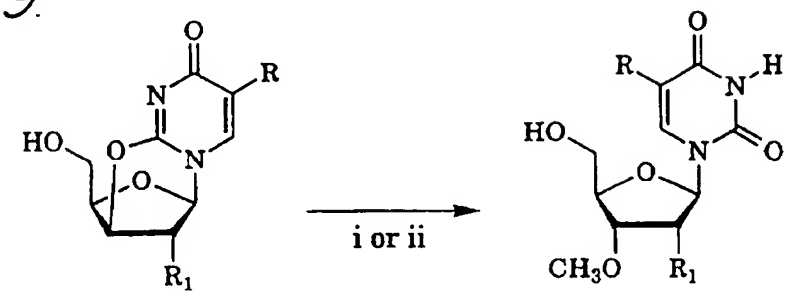


4 5

iii. a. $B(OCH_3)_3$ / MeOH
b. Ac_2O / DMF

iv. a. $B(OCH_3)_3$, BF_3•MeOH / MeOH
b. Ac_2O / DMF

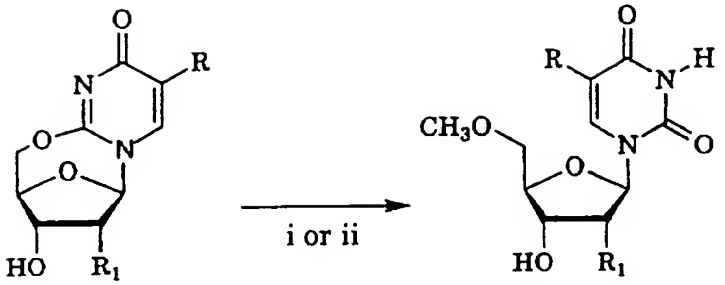
FIG. 9.



6 7

R=H, CH_3
R_1=OH, H

i. $B(OCH_3)_3$, BF_3•MeOH / MeOH
ii. $B(OCH_3)_3$ / MeOH



8 9

i. $B(OCH_3)_3$, BF_3•MeOH / MeOH
ii. $B(OCH_3)_3$ / MeOH

R=H, OCH_3
R_1=OH, H

FIG. 10.

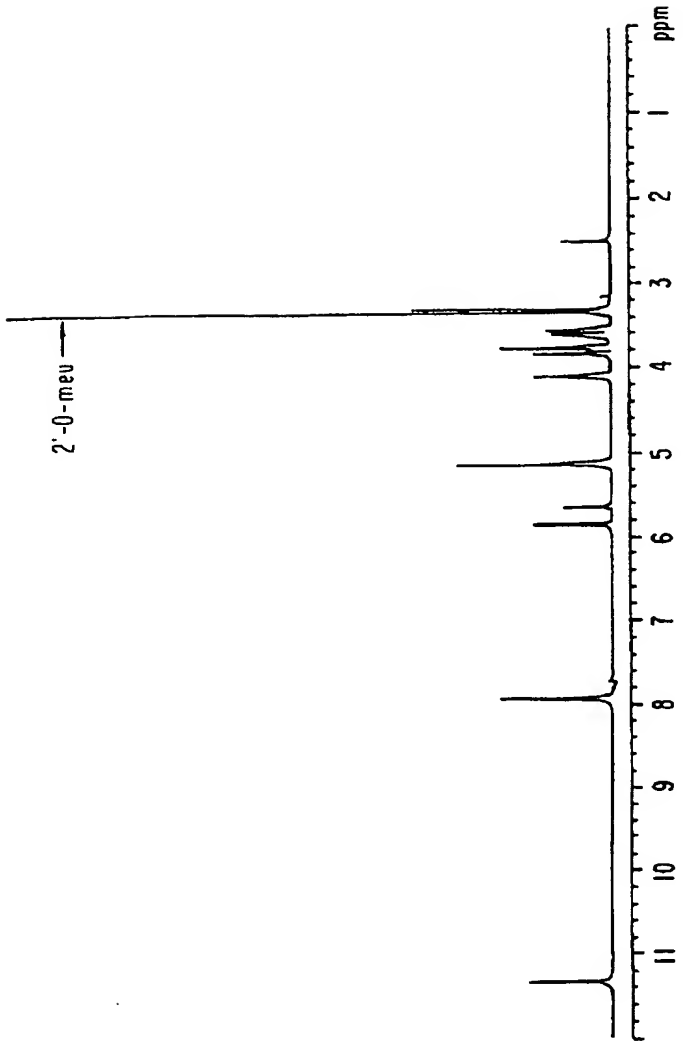


FIG. 11A.

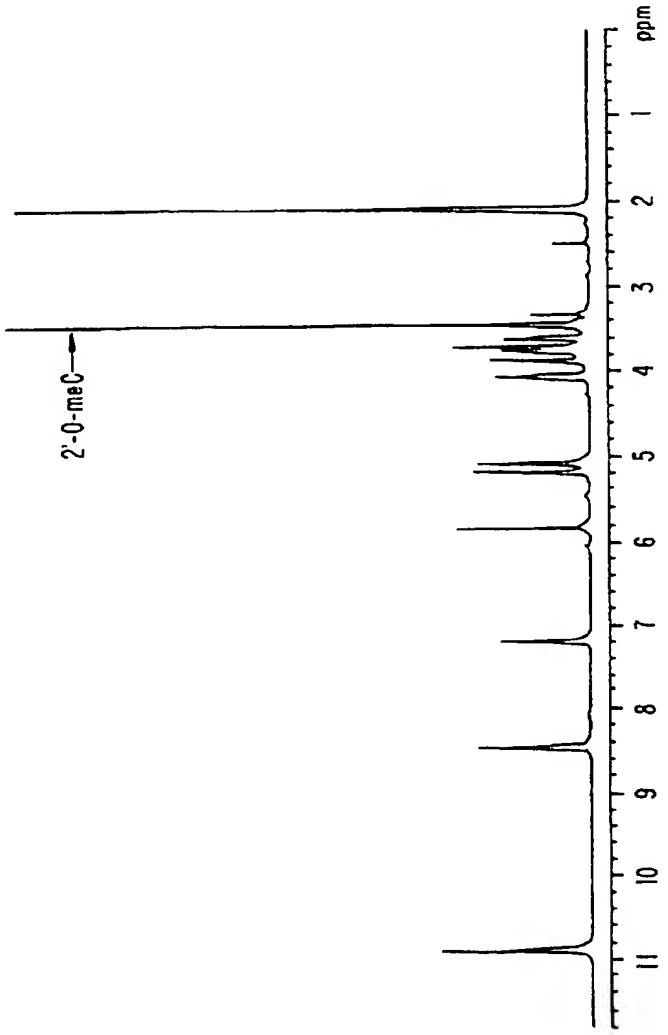
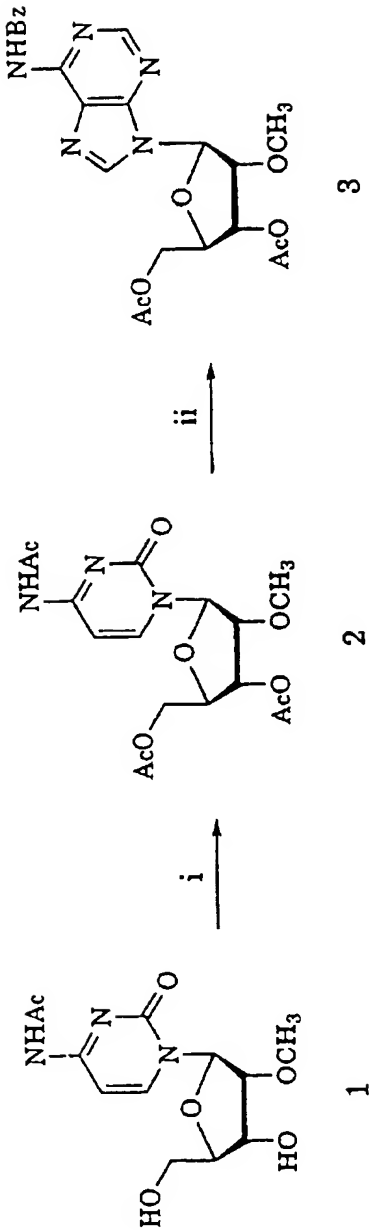
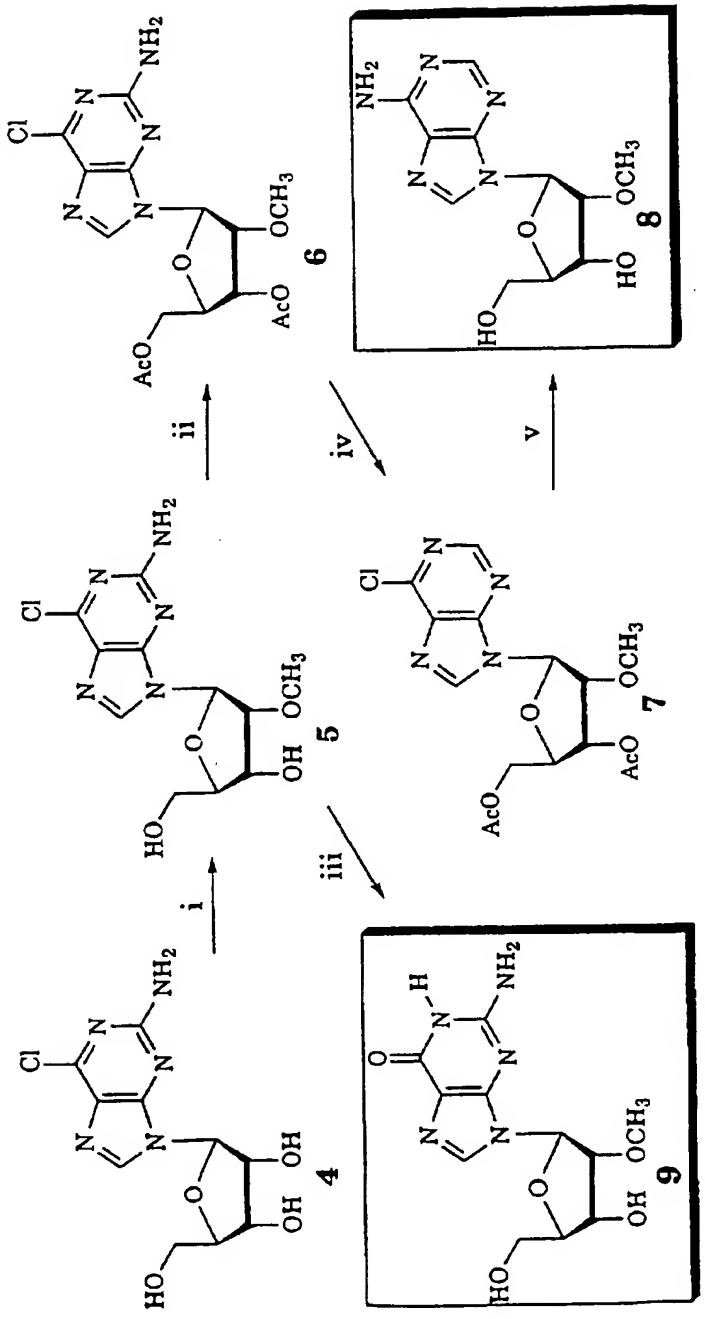


FIG. 11B.



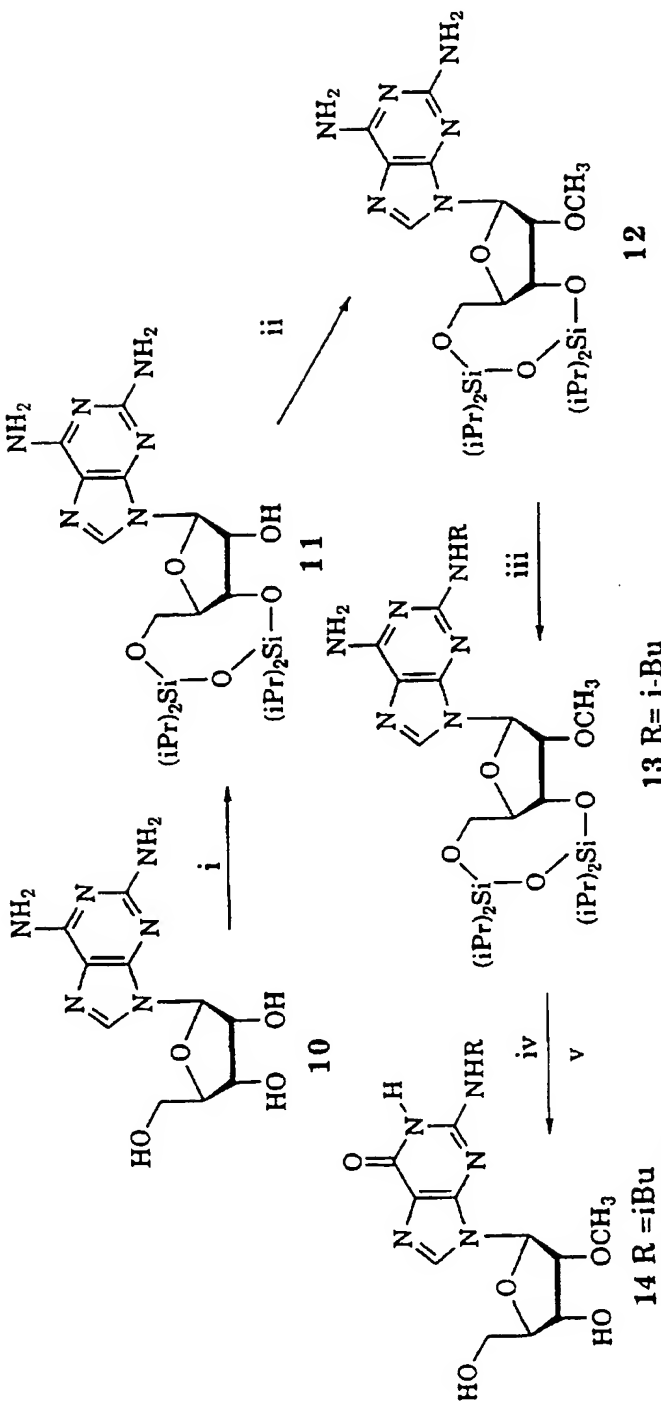
i. Ac_2O, pyridine/DMF
ii. N^6-benzoylaminopurine, BSA, TMStriflate/MeCN

FIG. 12.



i) NaH/ MeI/DMF; ii) Ac_2O, DMAP, Et_3N/ CH_3CN; iii) DABCO/water;
iv) isoamylnitrite/THF; v) NH_3/MeOH

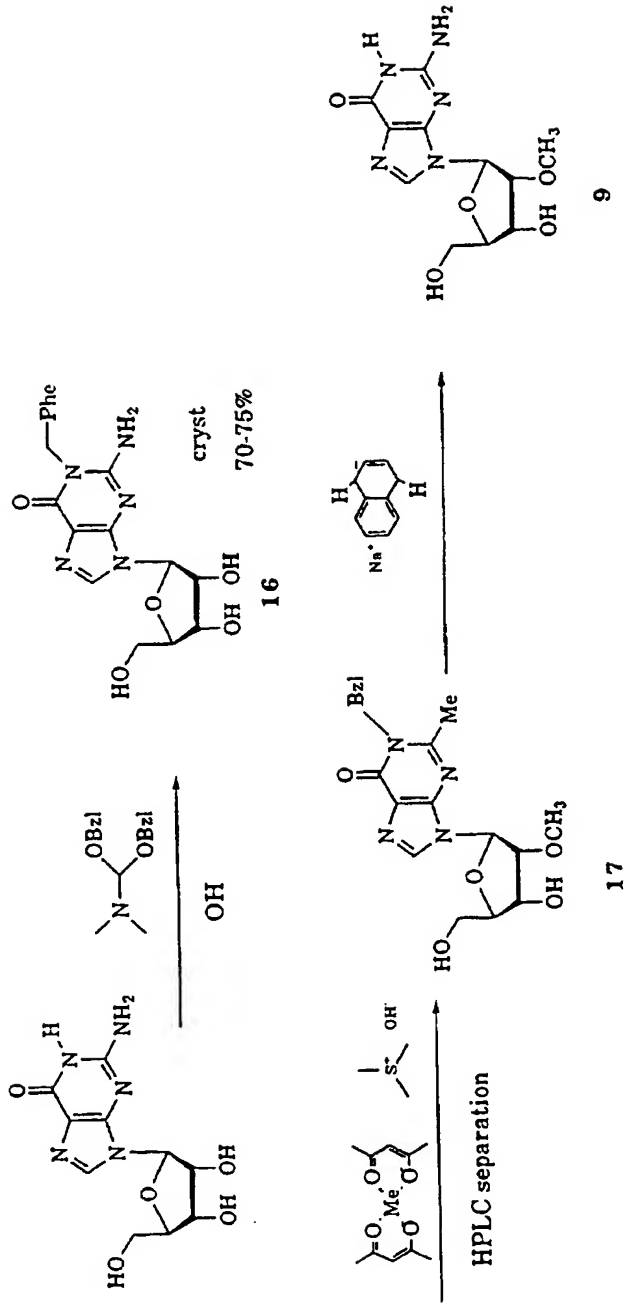
FIG. 13.



i. TIPSCl/pyridine; ii. MeI, NaH/DMF 0°C; iii. iPrPACCl/pyridine or iBuCl/pyridine; iv. HOAc, $NaNO_2/H_2O$; v. TEA•3HF

FIG. 14.

FIG. 15.



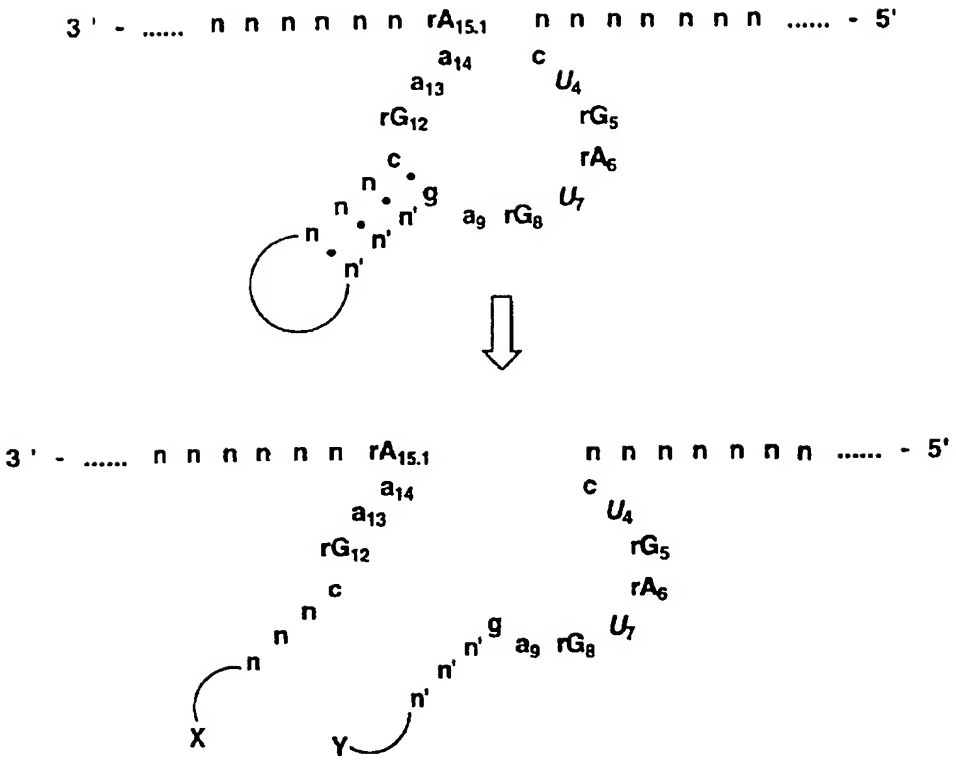
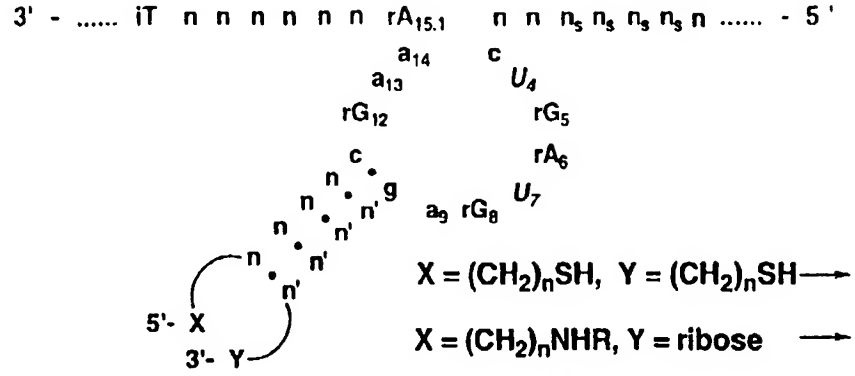

3' - ..... n n n n n n rA15.1 n n n n n n n ..... - 5'
a14
c
a13
U4
rG12
rG5
c
rA6
n
g
n
n'
U7
n
n'
a9 rG8
n'
3' - ..... n n n n n n rA15.1 n n n n n n n ..... - 5'
a14
c
a13
U4
rG12
rG5
c
rA6
n
n
g
n
n'
a9 rG8
U7
n
n'
n'
X
Y

FIG. 16.

FIG. 17



NOTE: $(CH_2)_n$ refers to any linkage. In addition, X and Y can be interchanged.

X = $(CH_2)_nSH$, Y = $(CH_2)_nSH$ → disulfide

X = $(CH_2)_nNHR$, Y = ribose → morpholino

X = $(CH_2)_nNHR$, Y = CO_2H → amide

X = $(CH_2)_nX$, Y = $(CH_2)_nOH$ → ether, X = halogen

X = $(CH_2)_nNHR$, Y = CHO → amine

X = $(CH_2)_nPPh_3$, Y = CHO → double bond

X = $(CH_2)_nNHR$, Y = $(CH_2)_nSO_2Cl$ → sulfonamide

X = $(CH_2)_nOH$, Y = CO_2H → ester

X = $(CH_2)_nX$, Y = $(CH_2)_nSH$ → thioether, X = halogen

X = $(CH_2)_nCOX$, Y = $(CH_2)_nOH$ → carbonate, X = halogen

X = $(CH_2)_nCOR$, Y = $(CH_2)_nRNH$ → hydrazone

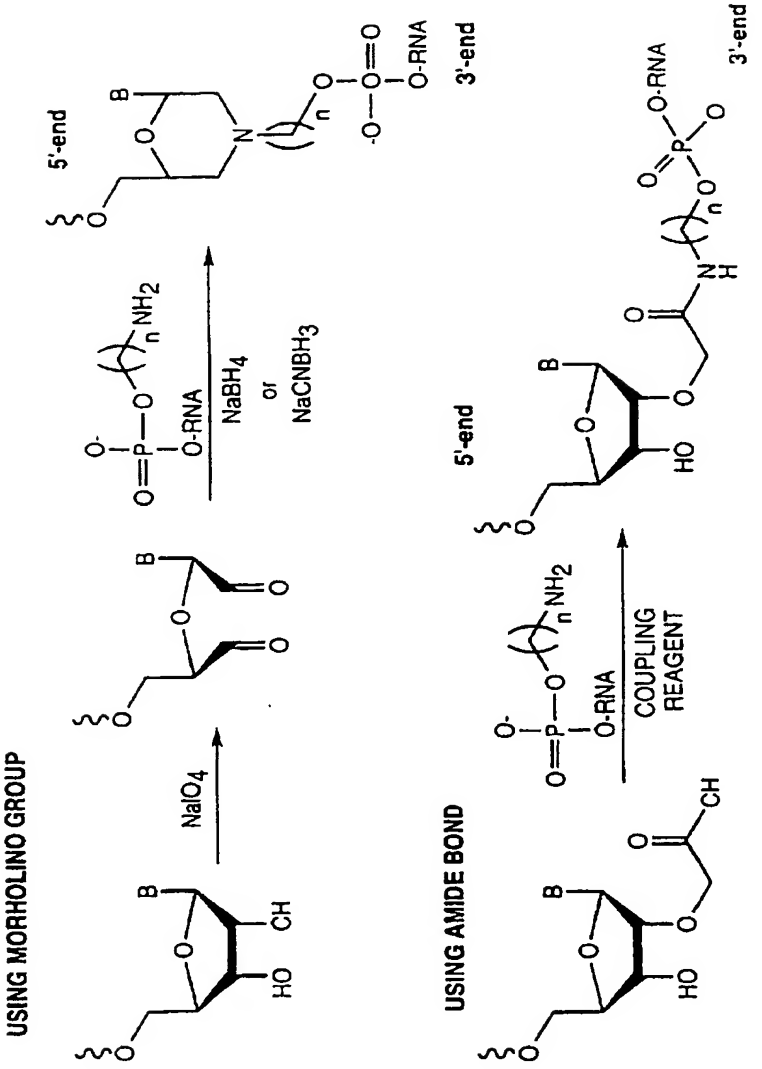

USING MORHOLINO GROUP
USING AMIDE BOND
5'-end
3'-end
NaIO4
NaBH4
or
NaCNBH3
O-RNA
COUPLING
REAGENT

FIG. 18.

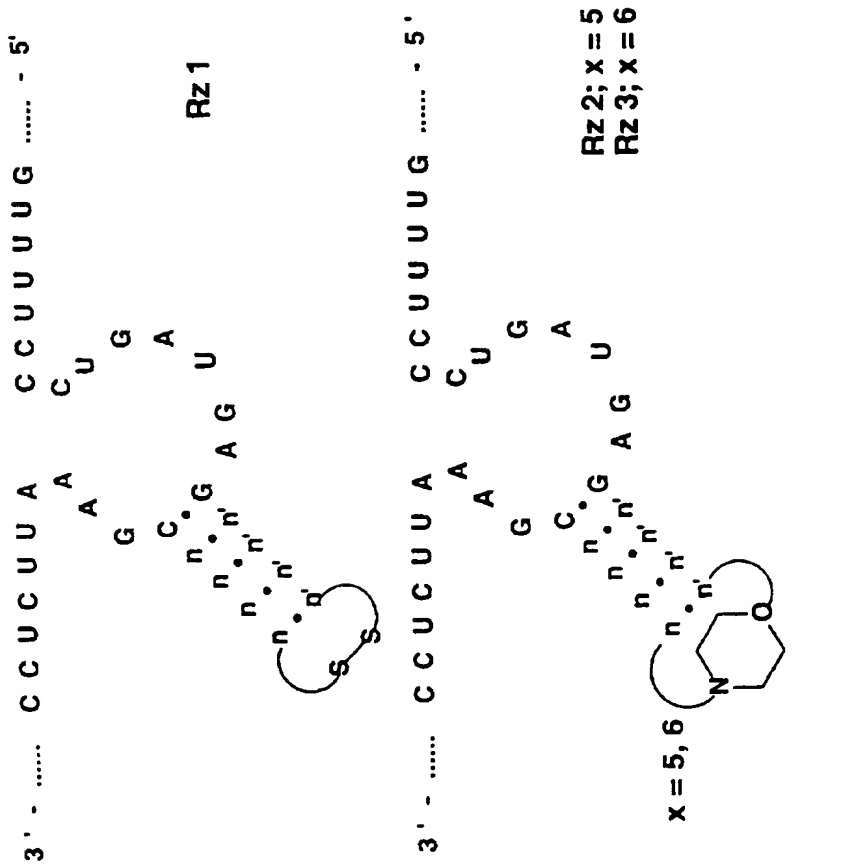


FIG. 19.

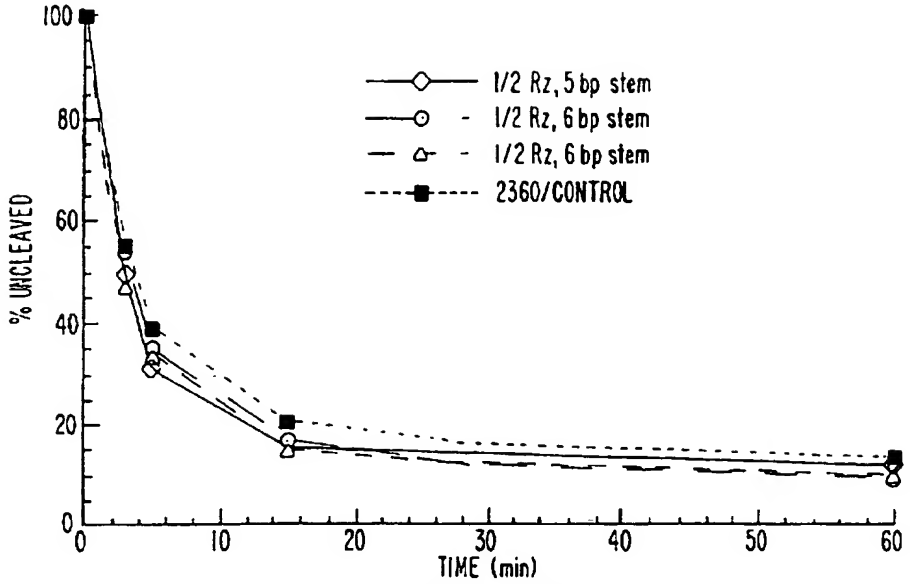

100
80
60
40
20
0
% UNCLEAVED
0
10
20
30
40
50
60
TIME (min)
1/2 Rz, 5 bp stem
1/2 Rz, 6 bp stem
1/2 Rz, 6 bp stem
2360/CONTROL

FIG. 20.

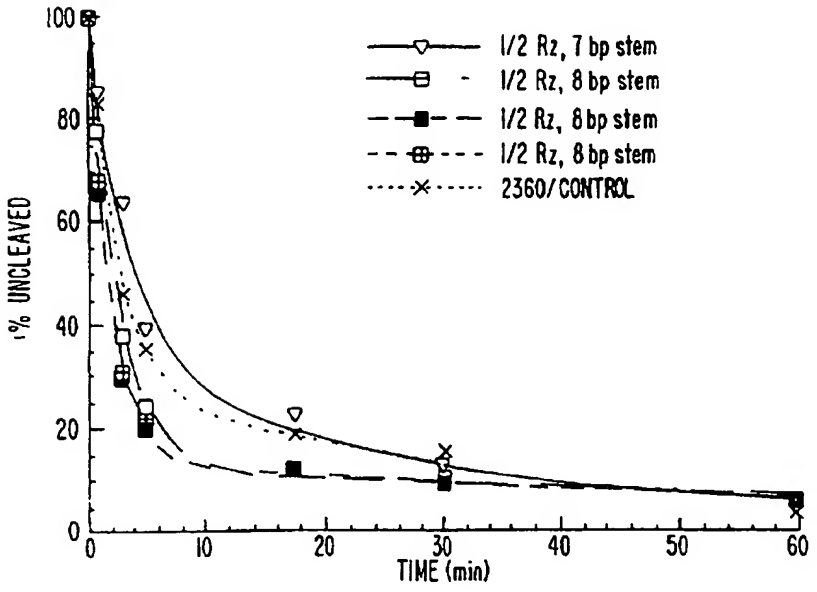

100
80
60
40
20
0
% UNCLEAVED
0
10
20
30
40
50
60
TIME (min)
1/2 Rz, 7 bp stem
1/2 Rz, 8 bp stem
1/2 Rz, 8 bp stem
1/2 Rz, 8 bp stem
2360/CONTROL

FIG 21

FIG. 22.

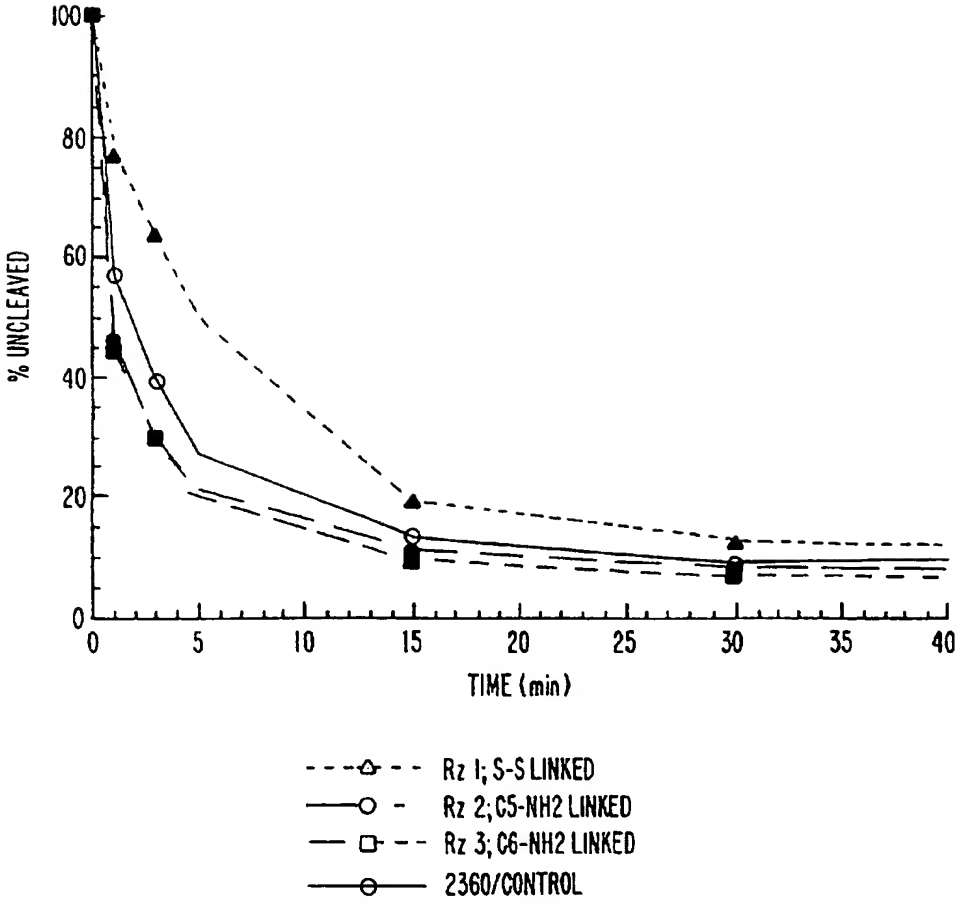
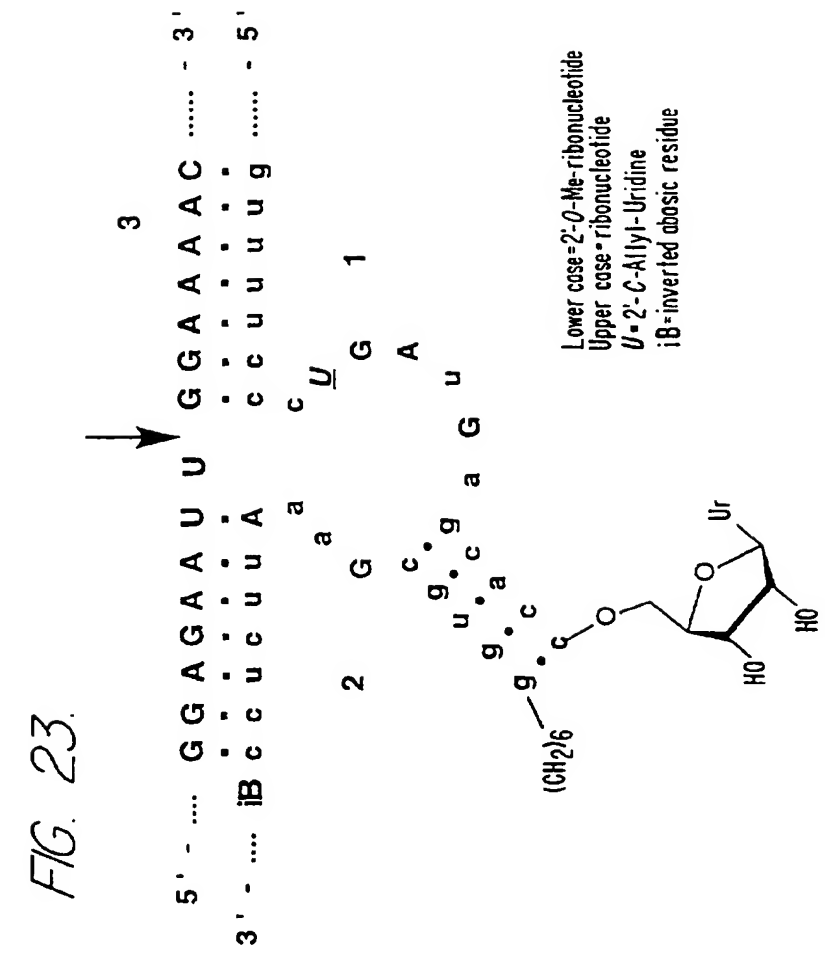

100
80
60
40
20
0
% UNCLEAVED
0
5
10
15
20
25
30
35
40
TIME (min)
Rz 1; S-S LINKED
Rz 2; C5-NH2 LINKED
Rz 3; C6-NH2 LINKED
2360/CONTROL

FIG. 23


5' - .... GGAGAAUU GGAAAAC ..... - 3'
3' - .... iB c c u c u u A c c u u u u g ..... - 5'
3
2 G a G c U G A 1
g • c
u • a
g • c
g • c
(CH2)6
O
O
Ur
HO
HO
Lower case=2'-O-Me-ribonucleotide
Upper case=ribonucleotide
U=2'-C-Allyl-Uridine
iB=inverted abasic residue

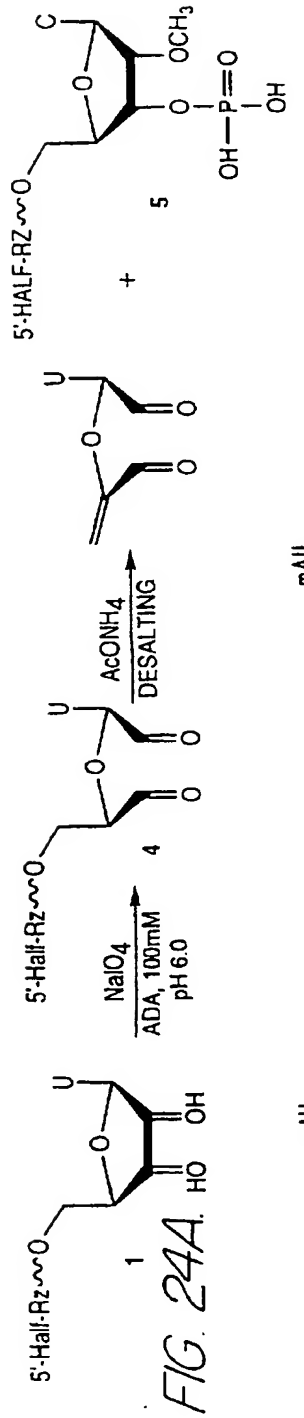


FIG. 24A.

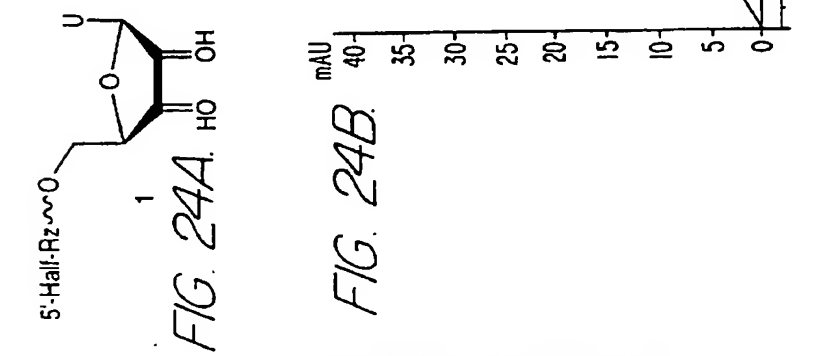


FIG. 24B

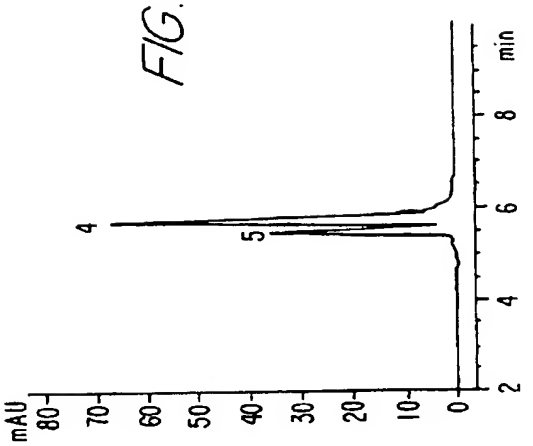


FIG. 24C

(A) OXIDATIVE CLEAVAGE AND E_2 ELIMINATION REACTION OF 1. (B) ANION-EXCHANGE HPLC ANALYSIS OF RECTION OF 1 WITH $NaIO_4$ AT RT AFTER 0, 30 AND 120 MIN. (C) ANION-EXCHANGE HPLC ANALYSIS OF AMMONIUM ACETATE DESALTED 4.

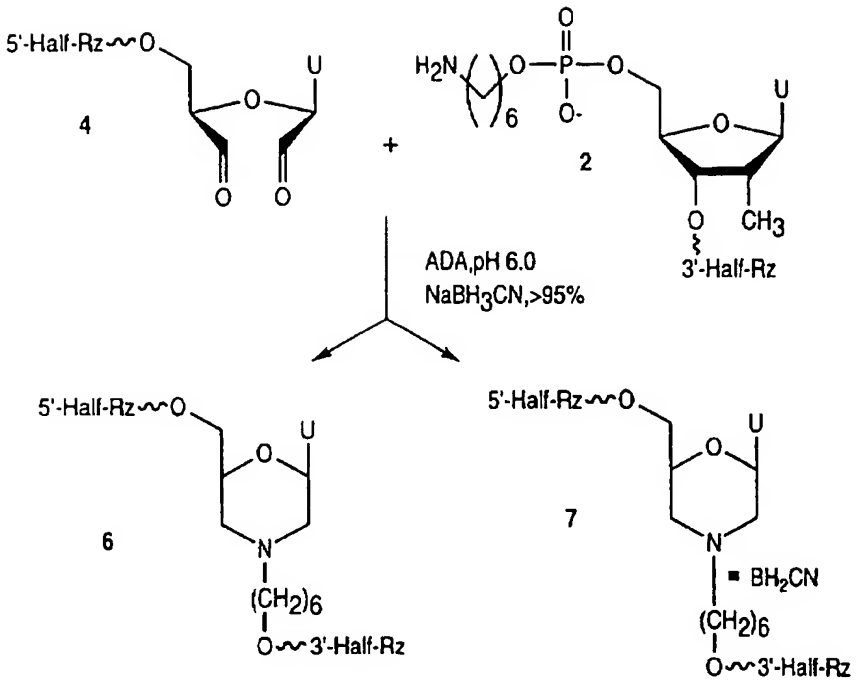
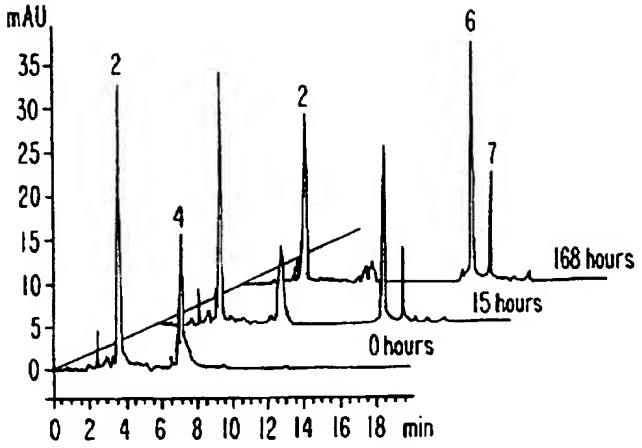

5'-Half-Rz
O
U
4
+
H_2N
6
O-
2
CH_3
3'-Half-Rz
ADA,pH 6.0
$NaBH_3CN$,>95%
N
$(CH_2)_6$
6
7
BH_2CN

FIG. 25.



ANION-EXCHANGE HPLC ANALYSIS OF THE REDUCTIVE ALKYLATION OF 2 AND 4 AFTER 0, 15 AND 168 HOURS.

FIG. 26.

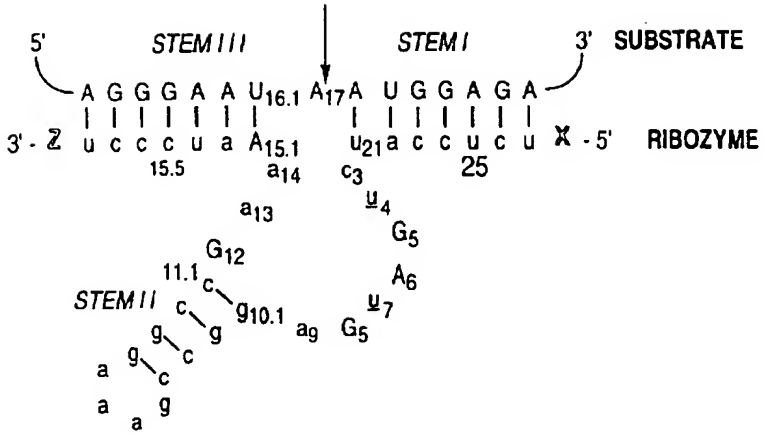
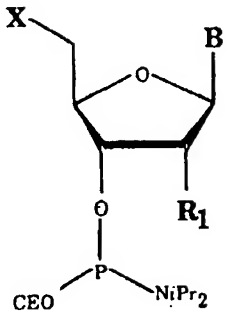

5'
STEM III
STEM I
3'
SUBSTRATE
A G G G A A U16.1 A17 A U G G A G A
3' - Z u c c c u a A15.1 u21 a c c u c u X - 5'
RIBOZYME
15.5
a14
c3
25
a13
u4
G5
G12
11.1
c
A6
STEM II
c
g10.1
u7
g
a9
G5
g
c
a
g
c
a
g
a

FIG. 27.

FIG. 28A.



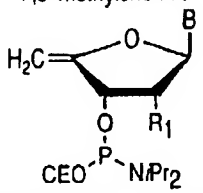
X = H, alkyl, MMTrNH-alkyl, DMTO-alkyl, Hal, $CHal_3$, NHMMTr, NHR, NR_2, NO_2, $CONH_2$, COOR, STr, SR-alkyl, OR, N_3, ONHR, or ONR_2

B = Natural bases, Modified bases or H

$\mathbf{R_1}$ = H, O-Alkyl, C-alkyl, OTBDMSi, Hal, NHR (R = protecting group), or OCH_2SCH_3

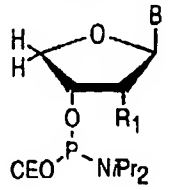
5'-modified sugars syntheses: Moffatt, J.G. In *Nucleoside Analogues: Chemistry, Biology and Medical Applications,* Walker, R.T. De Clercq E., Eckstein, F., Eds.; Plenum Press: New York, 1979, pp 71.

4',5' methylene PA

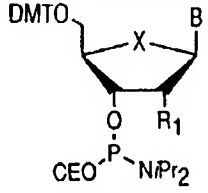


J. Am Chem. Soc. 1966, 88,5684
(nucleoside synthesis)

β-D-*Erythro*furanosyl Nucleoside PA



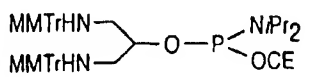
4'-thio,carbocylic PA



X=S, or CH_2

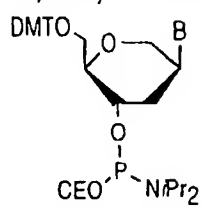
Nucleic Acids Res. 1993,21, 1587

N,N'-Bis(4-monoethoxytrityl)-1,3-diamino-2-propyl PA



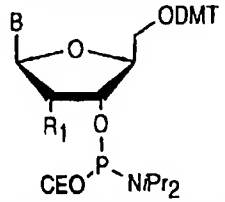
J. Med. Chem. 1995, 38, 4587

1,5-anhydrohexitol PA



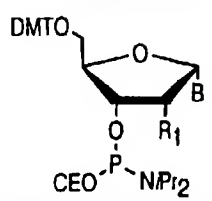
J. Med. Chem. 1995, 38, 826

L-nucleosides PA



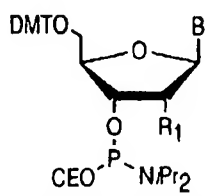
J. Am Chem. Soc. 1992, 114, 9731

α-nucleosides PA



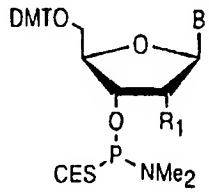
Antisense Res. Dev. 1994, 4, 251

Modified Base PA



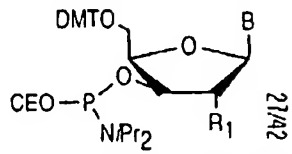
B=H,(4)-Pyridon, 6-Aza(Me)-pyrimidine, C-Phenyl,N7-Me-guanine, N-Me-imidazole) or substituted imidazole),pyridine (or substituted pyridine)- other modified bases

dithioate PA



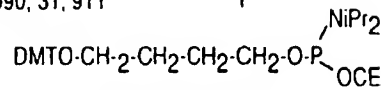
Tetrahedron Lett. 1990, 31, 911

β-D-*threo*-pentofuranosyl PA



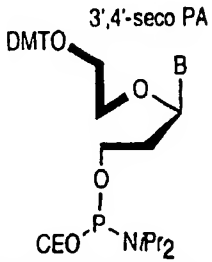
Bioorg. Khim. 1994,20,669

R_1=H



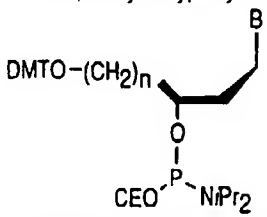
1,4-Butanediol PA

3',4'-seco PA



Nucleic Acids Res. 1984, 22, 703

n=1: 3,4-dihydroxybutyl PA
n=2: 3,5-dihydroxypentyl PA



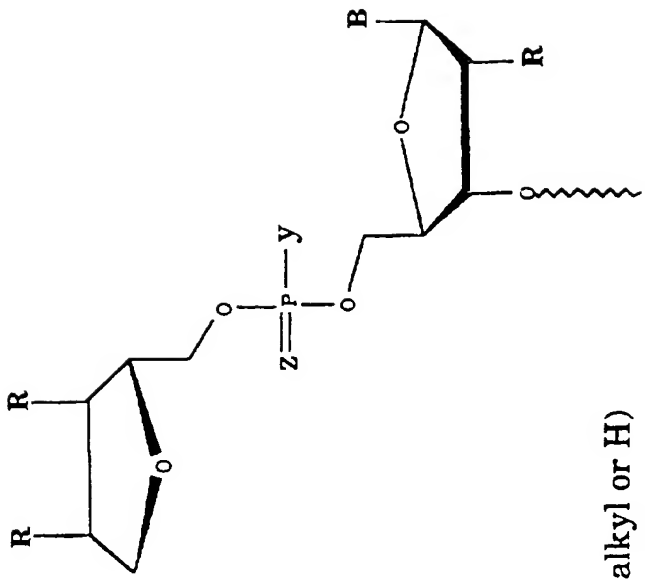
Nucleic Acids Res. 1984, 22, 703
Tetrahedron 1993, 19, 7223

COMMERCIALLY AVAILABLE

PA=2-Cyanoethyl N,N-diisopropyl phosphoeamidite

FIG. 28B

FIG. 28C



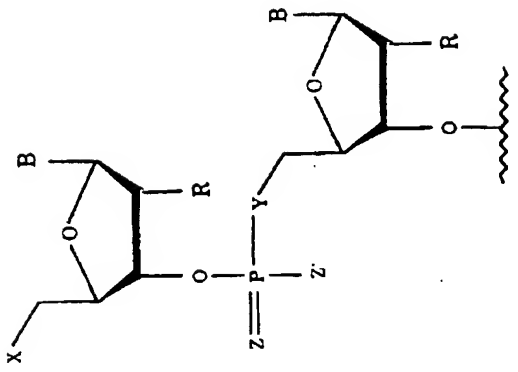
z = O or S

y = O, S or NHR_1 (R_1 = alkyl or H)

R = H, 2'-*O*-Alkyl, 2'-*C*-Alkyl, Hal, NHR_1 (R_1 = H, alkyl, aryl, acyl), or O-CH_2SCH_3

B = natural bases, modified bases or H

FIG. 29A



X = OH, Y = NH, Z = O

X = NH_2, Y = O, Z = O

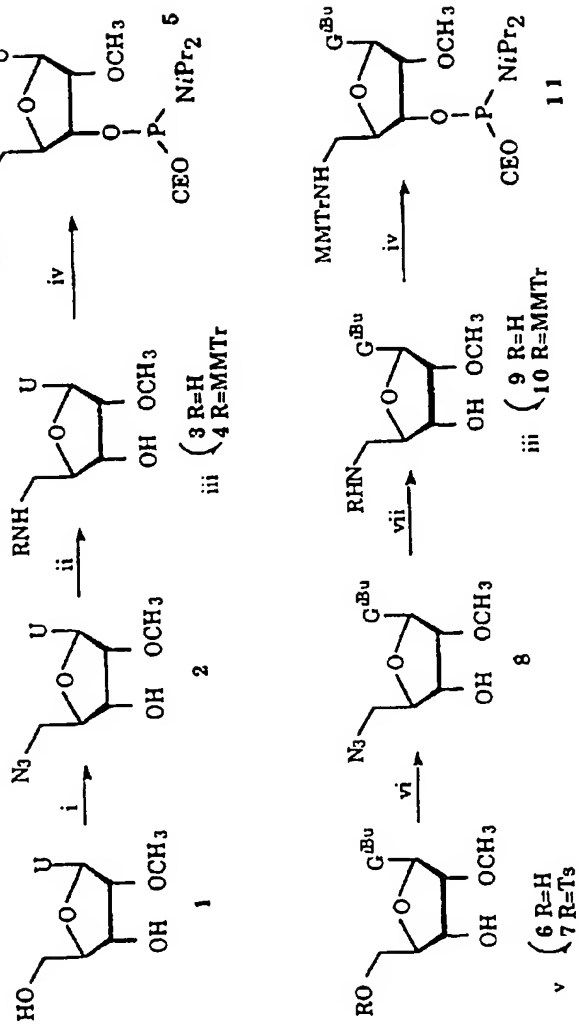
X = OH, Y = S, Z = O

X = OH, Y = O, Z = S

R = H, 2'-*O*-Alkyl, O-Si, 2'-*C*-Alkyl, Hal, NHR_1 (R_1 = H, alkyl, aryl,acyl), or O-CH_2SCH_3

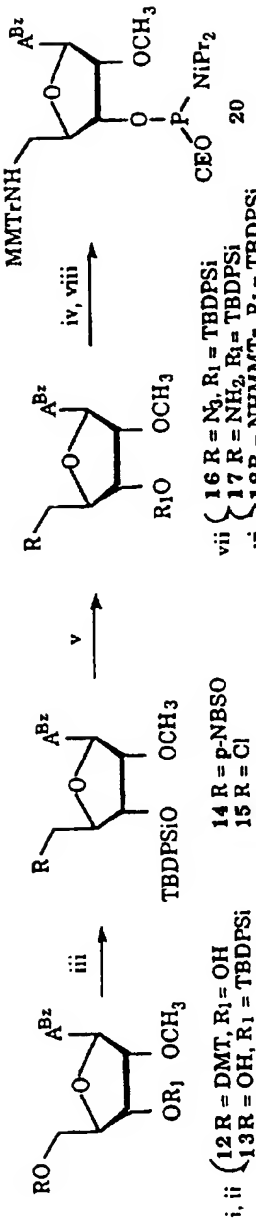
B = natural bases, modified bases or H

FIG. 29B



Reagents:
i, $Ph_3P/CBr_4/LiN_3/DMF$; ii, $Ph_3P/Pyr/NH_4OH$; iii, MMTr-Cl/DMAP/Et_3N/Pyr; iv, P(OCE)(NiPr_2)Cl/DIPEA/CH_2Cl_2;
v, Ts-Cl/Pyr; vi, LiN_3/DMF/55 °C; vii, 10% H_2/Pd-C/EtOH.

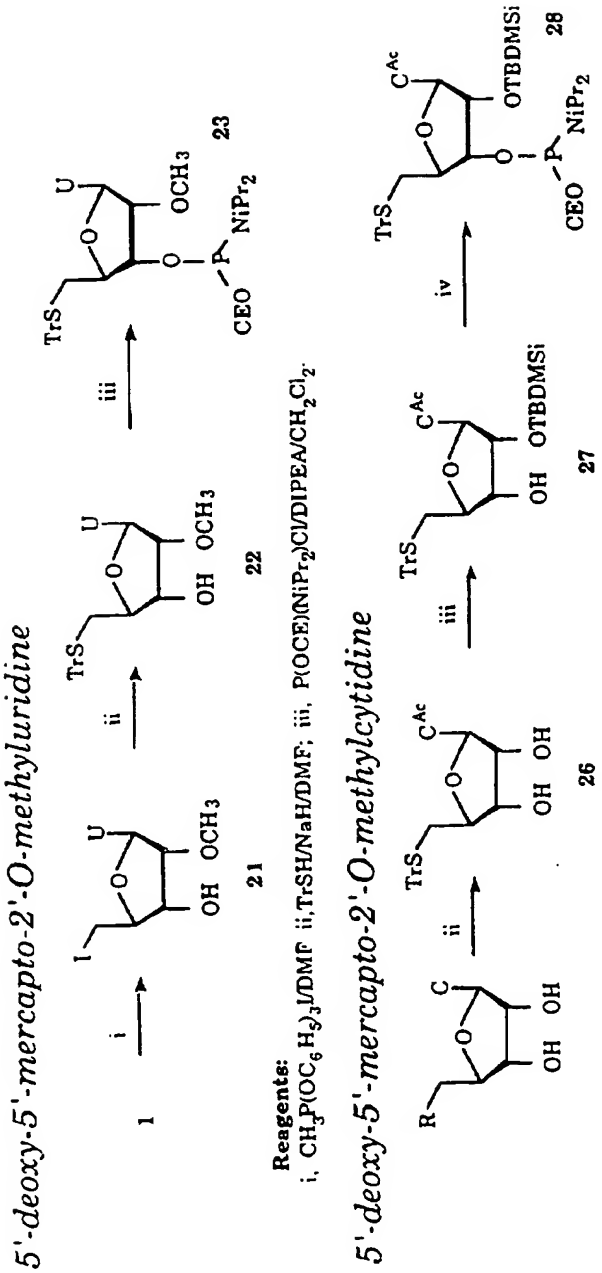
FIG. 29C



Reagents.

i, TBDPSi-Cl/Im/DMF; ii, TFA/DCM; iii, p-nitrobenzenesulfonyl chloride/Pyr; iv, TBAF/THF; v, LiN_3/DMF/55°C; vi, MMTr-Cl/DMAP/Et_3N/Pyr; vii, 10% H_2/Pd-C/EtOH; viii, P(OCEXNiPr_2)Cl/DIPEA/CH_2Cl_2.

FIG. 29D


5'-deoxy-5'-mercapto-2'-O-methyluridine
1
21
22
23
Reagents:
i, $CH_3P(OC_6H_5)_3I$/DMF ii,TrSH/NaH/DMF; iii, P(OCE)(NiPr$_2$)Cl/DIPEA/CH_2Cl_2.
5'-deoxy-5'-mercapto-2'-O-methylcytidine
24 R = Cl
25 R = TrS
26
27
28
Reagents:
i, TrSH/NaOH/reflux; ii, Ac_2O/EtOH/reflux; iii, TBDMSi-Cl/Py/Im; iv, P(OCE) (NiPr$_2$)Cl/DIPEA/1-MeIm/CH_2Cl_2.

INHIBITION OF SMOOTH MUSCLE CELL PROLIFERATION BY 5'- AND 3'-END-MODIFIED RIBOZYMES

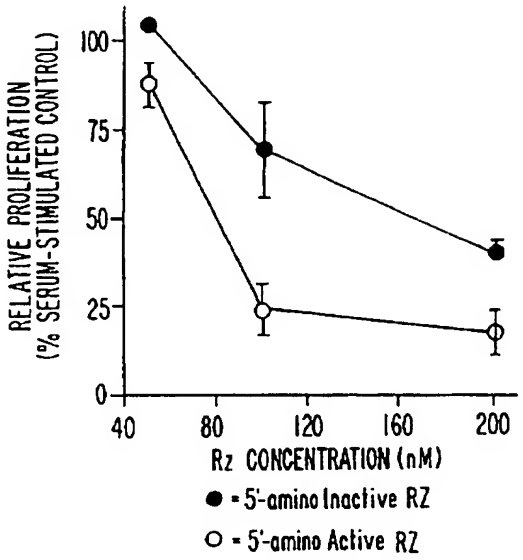


FIG. 30.

INHIBITION OF SMOOTH MUSCLE CELL PROLIFERATION BY 5'- AND 3'- END-MODIFIED RIBOZYMES

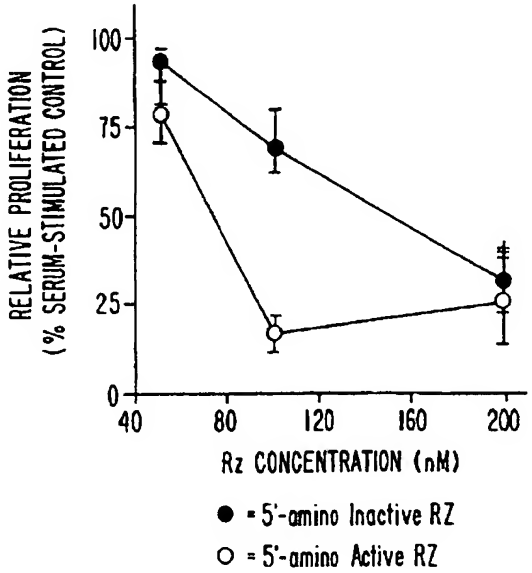
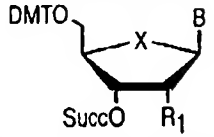


FIG. 31.

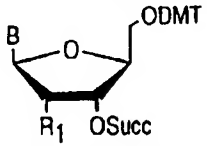
FIG. 32A

4'-thio,carbocylic



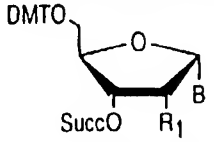
Nucleic Acids Res. **1993**,21, 1587

L-nucleosides



J. Amer. Chem. Soc. 1992, 114, 9731

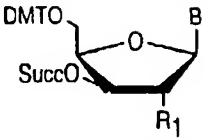
α-nucleosides PA



Antisense Res. Dev. 1994, *4*, 251

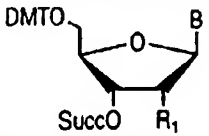
Succ=$CO(CH_2)_2COOH$

threo--pentofuranosyl



Bioorg. Khim. **1994**,20,669- for R_1=H

BASE MODIFICATION



B=H,2(4)-Pyridon, 6-Aza(Me)-pyrimidine, C-Pheny pyridine (or substituted pyridine)-other modified bases

Imbach *et al.*, 1994 *Nucleic Acids Res.* 22, 2183

DITHIOATE

Tetrahedron Lett. **1990**, 31, 911.

1,4-butanediol

3'-3'-inverted abasic ribose or deoxribose

3'-2'-inverted abasic ribose or deoxribose

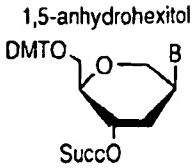
Bridging methylphosphonate

n=6: hexanol

$DMTO-(CH_2)_nO-CO(CH_2)_2CONH-CpG$

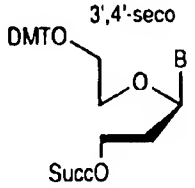
COMMERCIALLY AVAILABLE

1,5-anhydrohexitol



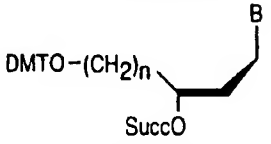
J. Med. Chem. **1995**, 38, 826

3',4'-seco



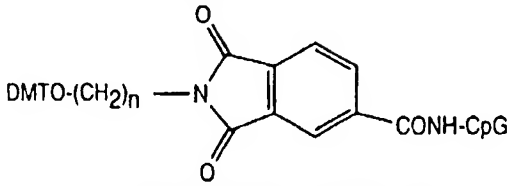
Nucleic Acids Res. **1994**, 22, 703

n=1: 3,4-dihydroxybutyl
n=2: 3,5-dihydroxypentyl



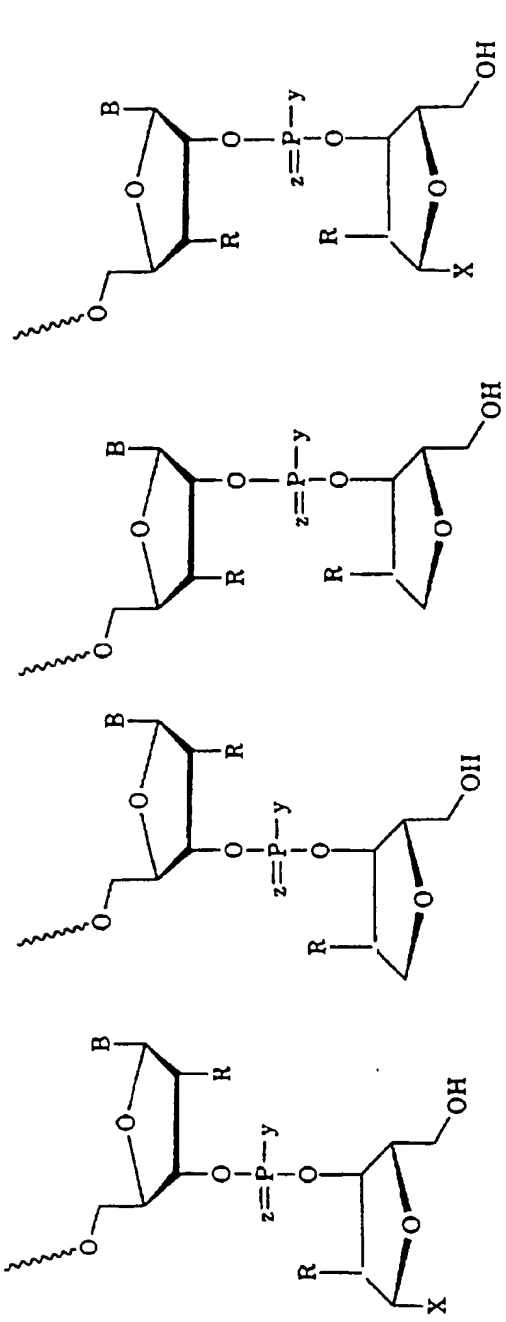
Tetrahedron 1993, 19, 7223
Nucleic Acids Res. **1991**, 19, 2587

n=6: aminohexyl



Nucleic Acids Res. **1993**,21.145
n=1-6

FIG. 32B



3'-3'-inverted nucleotide 3'-3'-inverted Abasic 3'-2'-inverted abasic 3'-2'-inverted Nucleotide

B = natural bases, modified bases, or H

R = H, halo, NHR_1 (R_1 = H, alkyl, aryl, acyl), alkyl, O-alkyl, OCH_2SCH_3, or other related modifications

X = natural or modified base

y = O, S or NHR_1 (R_1=alkyl or H); z = O or S

FIG. 33.

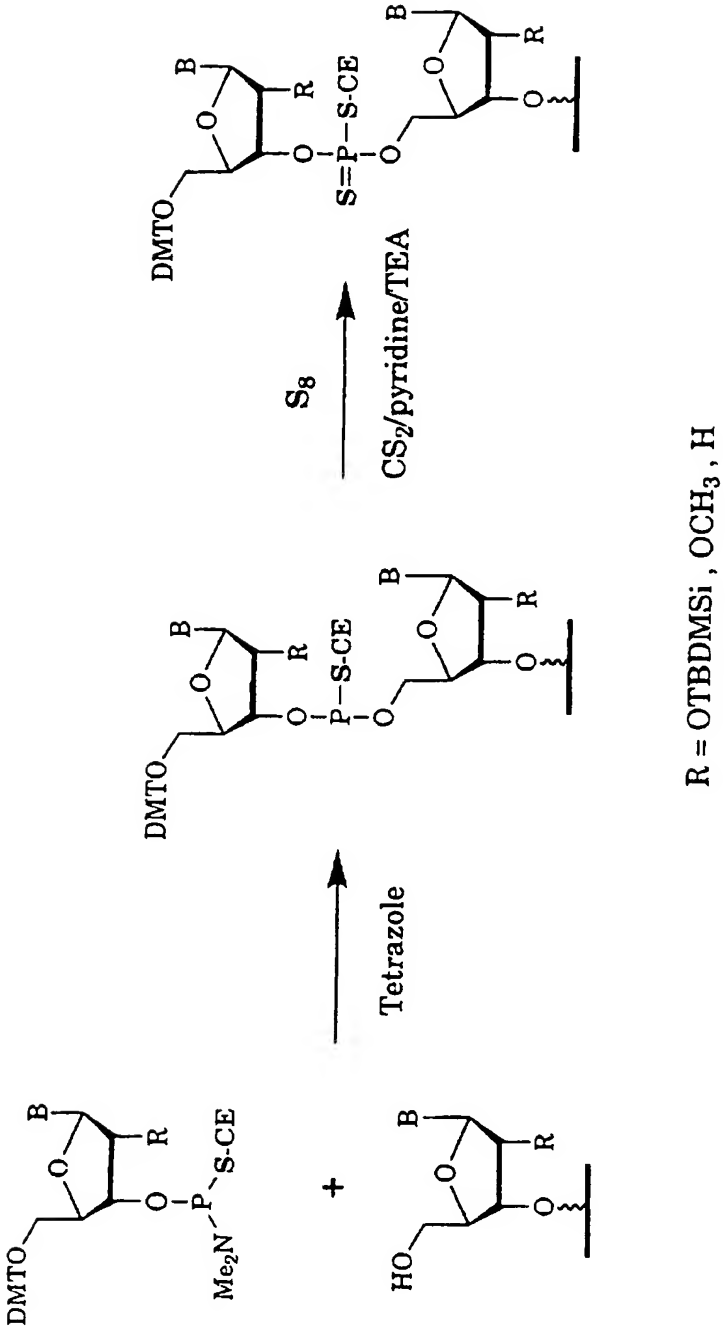

DMTO
B
O
R
O
P
S-CE
Me2N
HO
Tetrazole
S8
CS2/pyridine/TEA
S=P-S-CE
R = OTBDMSi , OCH3 , H

FIG. 34

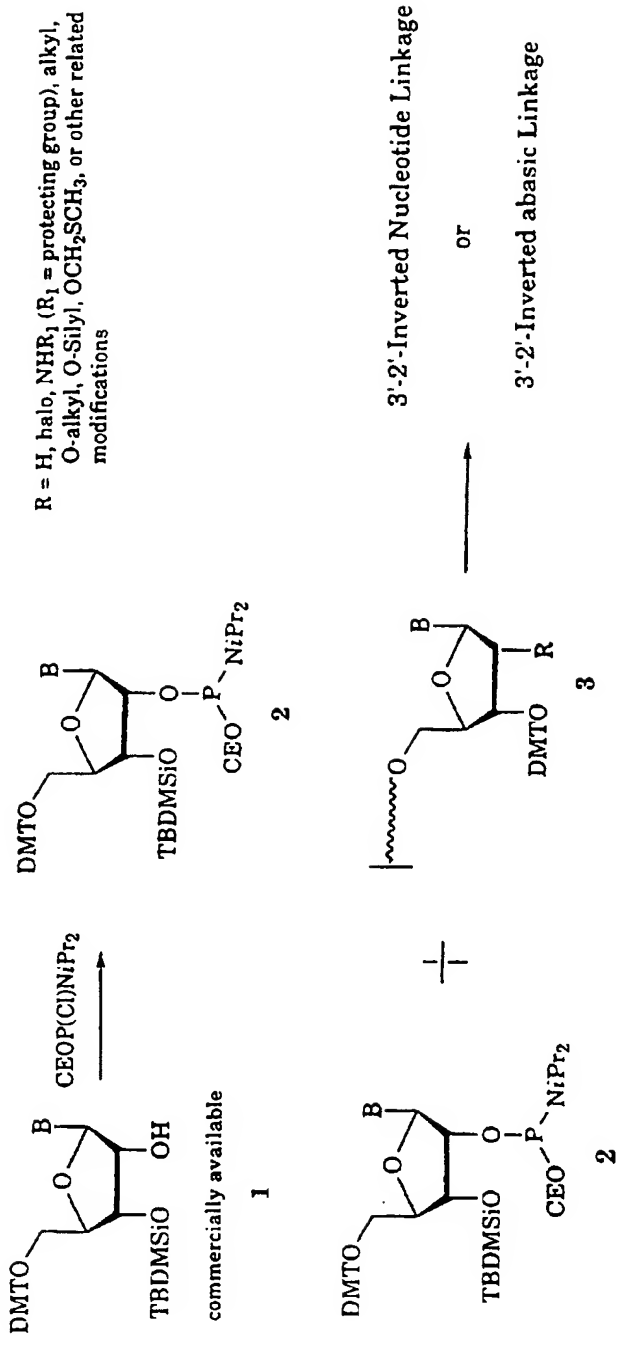
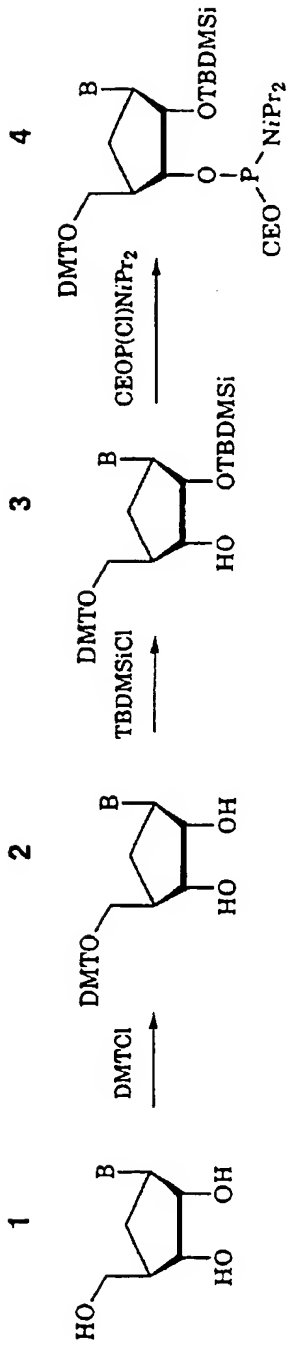

B = natural bases, modified bases or H
R = H, halo, NHR1 (R1 = protecting group), alkyl, O-alkyl, O-Silyl, OCH2SCH3, or other related modifications
CEOP(Cl)NiPr2
commercially available
1
2
3
3'-2'-Inverted Nucleotide Linkage
or
3'-2'-Inverted abasic Linkage
DMTO
TBDMSiO
OH
CEO
NiPr2
B
R

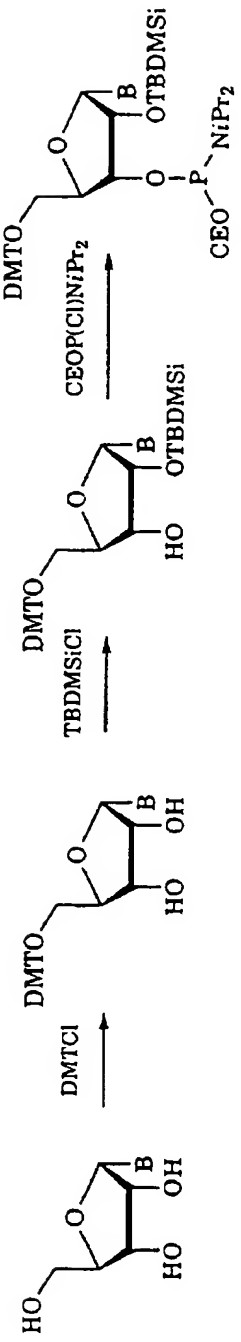
FIG. 35



Tetrahedron **1994**, *50*, 10611.

B = natural bases, modified bases or H

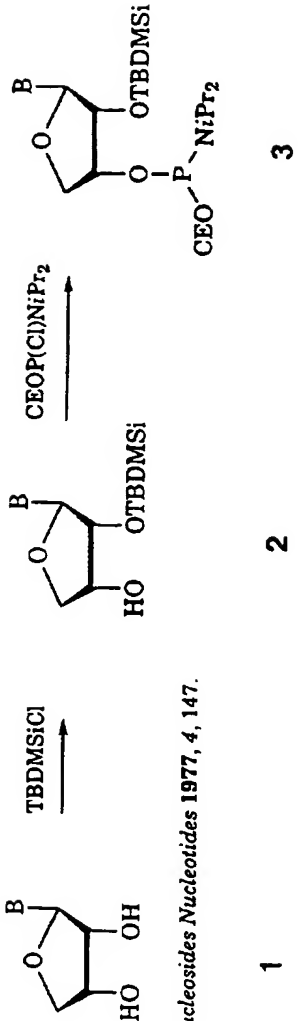
FIG. 36



Nucleic Acid Res. 1992 20,1193

B = natural bases or modified bases

FIG. 37

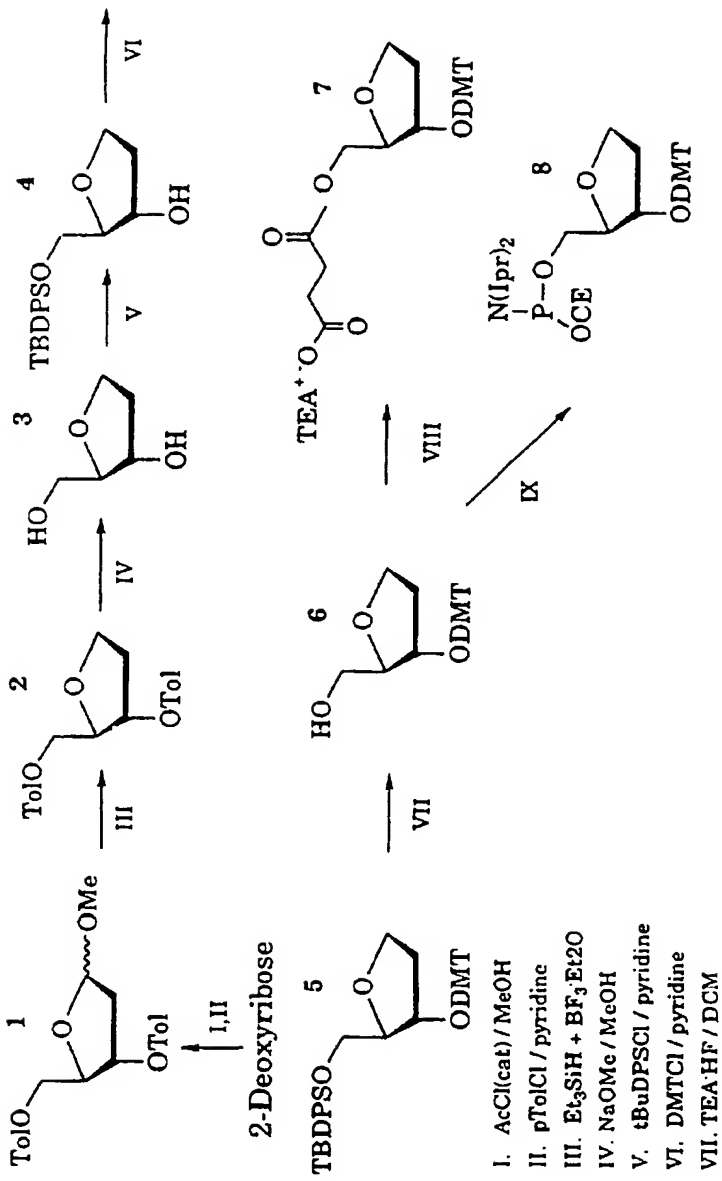


1 2 3

B = natural bases or modified bases

J. Carbohydr. Nucleosides Nucleotides **1977**, *4*, 147.

FIG. 38



I. AcCl(cat) / MeOH
II. pTolCl / pyridine
III. $Et_3SiH + BF_3Et2O$
IV. NaOMe / MeOH
V. tBuDPSCl / pyridine
VI. DMTCl / pyridine
VII. TEA·HF / DCM
VIII. Succinic Anhydride + DMAP
IX Phosphitylation

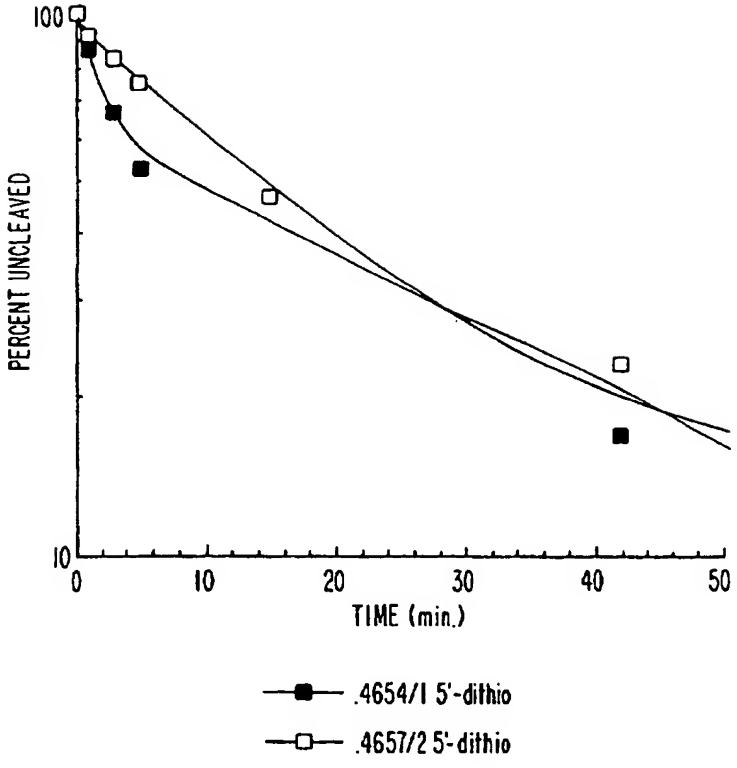

100
10
PERCENT UNCLEAVED
0
10
20
30
40
50
TIME (min.)
.4654/1 5'-dithio
.4657/2 5'-dithio

FIG. 39.